ANGIOTENSIN CONVERTING ENZYME AND INTERLEUKIN-6, GENETIC POLYMORPHISMS AND THEIR RELATION TO SURGICAL OUTCOME

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ABSTRACT

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Improving surgical outcome by identifying the high-risk patient has traditionally concentrated on the ability to *tolerate* the insult of surgery. There has been increasing interest in the inflammatory *reaction* to surgery, as a potential outcome predictor. Moreover, the considerable observed variation in the nature and scale of this response may be due to genotypic differences in functional cytokine polymorphisms. Thus, the Interleukin-6 (IL6) and Angiotensin-1-Converting Enzyme (ACE) expression responses to surgery were examined, along with possible association with outcome, and the influences of common functional polymorphisms (-174 G/C and I/D respectively).

Twenty nine elective colorectal cancer patients were recruited. From separated peripheral blood mononuclear cells, nucleic acids were extracted. PCR of DNA was used for genotyping, and a semi-quantitative RT-PCR of RNA method for analysing gene transcription used, including an assessment of the differential I/D allelic contribution to ACE transcription. Plasma protein expression was established by ELISA. Patients were prospectively followed, with morbidity and length of stay (LOS) chosen as end-points.

There was evidence of a post-operative increase (24 hour timepoint) in both IL6 gene transcription ($p=0.077$) and protein expression ($p<0.001$), but their extent associated with neither the -174 G/C polymorphism nor LOS. However, there was a trend towards C-allele carriers ($p=0.077$) and those launching a greater IL6 protein response ($p=0.078$) being less likely to experience complications.

Considering ACE I/D heterozygotes, the D-allele was responsible for greater levels of gene transcription than the I-allele, both pre- ($p<0.001$) and post-operatively ($p=0.01$; 24 hour timepoint). Overall, there was a post-operative rise in ACE gene transcription ($p=0.042$) with D/D homozygotes producing the greatest response ($p=0.002$), and D-allele carriers having a prolonged LOS. Conversely, plasma ACE protein levels fell post-operatively ($p<0.001$), and did not associate with I/D genotype or outcome.

Overall, this work contributes to previous observations that an assessment of patient genotype may aid the identification of the high-risk surgical patient.

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STATEMENT OF ORIGINALITY

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The work reproduced in this thesis was undertaken within the Department of Surgery at the Royal Free and University College London Medical School (Bloomsbury Campus), and is the sole of work of Mr Jason Lee.

CONTENTS

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ABBREVIATIONS

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AAA	Abdominal aortic aneurysm
ACE	Angiotensin-1-Converting Enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance between groups
ARDS	Adult respiratory distress syndrome
ASA	American society of anesthesiologists
AT	Angiotensin receptor
Bp	Base pair(s)
C-	Cytosine
CABG	Coronary artery bypass grafting
cDNA	Complementary DNA
CRP	C-Reactive protein
D-	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
5'	Five-prime
G-	Guanine
GAPDH-3	Glyceraldehyde-3-phosphate dehydrogenase
Hct	Haematocrit
I-	Insertion
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive care unit
IL1β	Interleukin-1 beta
IL6	Interleukin-6
IL10	Interleukin-10
IOD	Integrated optical density
kDa	Kilodalton
LOS	Length of stay
LVH	Left ventricular hypertrophy
MCP-1	Monocyte chemoattractant protein -1

Mg	Magnesium
MHC	Major histocompatibility complex
MI	Myocardial infarction
MOF	Multiple organ failure
mRNA	Messenger RNA
OD	*Optical density*
PBMN	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear cell
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
RT	Reverse transcription
S-JCA	Systemic onset juvenile chronic arthritis
s.e.m.	Standard error of mean
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
Taq	Thermus aquaticus
TGFβ	Transforming growth factor beta
Th	T-helper cell
TNFα	Tumour necrosis factor alpha
3'	Three-prime
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VO_2max	Maximal uptake of oxygen
WCC	White cell count

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction

CHAPTER 1

1.1 INFLAMMATION, SURGERY AND OUTCOME

1.1.1 Surgical Outcome and Identifying the High Risk Patient

Although surgical outcome may be defined in a wide variety of ways, it has now become virtually synonymous with post-operative morbidity and mortality. The ever increasing burden of public expectation allied with increasing media scrutiny means that surgeons are under greater pressure to achieve optimal outcomes from surgery.

Major surgery carries a significant risk of major post-procedural complications, and even death, particularly in patients who are elderly or with significant cardio-respiratory disease. For example, recent large audits in the UK have revealed a 30-day mortality rate of 5.6% for elective colorectal cancer surgery (Tekkis *et al.*, 2003), 7.3% for elective infrarenal aortic aneurysm and aorto-iliac occlusive disease surgery (Bayly *et al.*, 2001), 9–15% for oesophagectomy, 13–15% for elective gastrectomy (McCulloch *et al.*, 2003) and as high as 19.3% for emergency colorectal surgery (Tekkis *et al.*, 2003).

Outcome following major surgery is dependent on both controllable factors such as the medical care received before, during and after surgery, and the fixed factors of the patients physiological ability to tolerate surgical trauma and recover afterwards; the latter determined by a combination of genotype, and environmental influences. These intrinsic factors influencing outcome are largely beyond control, so regardless of the technical prowess of the surgeon and the excellence of post-operative care, some patients will still do badly. Much attention has focused on improving aspects of patient care that are firmly under the control of the surgical and nursing team; operative choice

and technique, antibiotic prophylaxis (Song *et al.*, 1998), thromoboembolic prophylaxis (Wille-Jorgensen *et al.*, 2003), and the introduction of medical emergency teams (Bellomo *et al.*, 2004) are just some of a multitude of examples. However, despite these measures and innovations, the outcome from major surgery has not improved as significantly as had once been hoped (Casson *et al.*, 2005). Different approaches are required in the quest to improve surgical outcome, and one of these has been to focus on identifying the high risk surgical patient. This group will have an operative risk which exceeds that associated with the natural progression of their disease. Accurate recognition of this high risk cohort will allow clinicians to modify their management appropriately - any ongoing medical treatment may be optimised, the anaesthetic plan of care altered and the planned surgical procedure modified. Most importantly though, the patient will be in a better position to assess their individual risk/benefit ratio for surgery and thus make a more informed decision on consenting for an operation, an especially important factor in light of the trend towards more aggressive surgery in older and more sick patients.

Identifying the high risk patient

The current strategies of identifying the high risk patient concentrate on an accurate pre-operative assessment that focuses on the physiological (cardiovascular and respiratory) ability to tolerate the stress of surgery. This is in turn based on the objective gathering of information on cardio-respiratory function, using tests such as lung function tests (Zibrak *et al.*, 1990), dobutamine stress echocardiography (Shaw *et al.*, 1996), dypyridamole thalium scintigraphy (Brown *et al.*, 1993), exercise electrocardiogram (ECG) (Gauss *et al.*, 2001) and cardiopulmonary exercise testing (Older *et al.*, 1999). These strategies are summarised in Table 1.1.

Assessment	End-point measure	Pros	Cons	Evidence
Exercise Electrocardiogram (ECG)	ECG ischaemic changes when performing treadmill exercise	Inexpensive and widely available	Requires patient to be able to exercise on treadmill, and can be limited by baseline ECG abnormalities	Gauss *et al*, (2001)
Lung function tests	Restrictive and obstructive lung abnormalities	Non-invasive, inexpensive and widely available	Static test that does not assess functional capacity	Gerson *et al*, (1990)
Dipyridamole thallium scintigraphy	Uses vasodilatory stress to identify areas of ischaemic myocardium	Good predictor of cardiac complications	Uses radio-active material, requires overnight fast and contra-indicated in asthma. Not effective as screening tool	Brown *et al*, (1998) Baron *et al*, (1994)
Dobutamine Stress Echocardiogram	Identifies ischaemic myocardium by motion wall abnormalities	Relatively cheap and non-invasive	Subjective test, only effective in medium/high risk patients and requires thoracic echogenicity	Shaw *et al*, (1999)
Cardiopulmonary exercise testing	Identifies anaerobic threshold and cardiac failure by measuring respiratory gases whilst performing graded exercise	Objective, non-invasive test that has proved predictive of cardiopulmonary complications	Not widely available, and efficacy has not really been demonstrated beyond a single centre	Older *et al*, (1999)

Table 1.1 Strategies employed in the pre-operative assessment of the high risk surgical patient

Regardless of the exact method used, such a stratification of risk then leads to a modification of peri-operative management, leading hopefully to improved outcome. A number of risk scoring systems have also been developed to quantify the severity of a patient's illness as a predictive measure for risk of morbidity or death. The most routinely used of these is the American Society of Anesthesiologists (ASA) grading, which aims to predict the risk of postoperative complications for a particular patient; indeed in combination with age, ASA grading has proved predictive of post-operative mortality (Hall *et al.,* 1996). However, the need for an advance in patient assessment is highlighted by the fact that 96% of patients who die within 30 days of surgery have significant medical co-morbidities at the time of surgery, of whom 42% are scored as ASA III or less (NCEPOD, 2002).

Rather than concentrating on the patient's ability to tolerate surgery, a novel approach may be provided by a greater understanding of the inherent *response* to such a major stress. An improved comprehension of the mechanisms and consequences of an individual patient's response to surgery will aid the identification of that cohort of pre-operative patients who are more likely to mount an abnormal (be that inadequate or excessive) response to surgery. When combined with current strategies for identifying existing pre-operative co-morbidity, significant improvements in surgical outcome may follow. More specifically, the response to surgery is the generic reaction of acute inflammation. So, before addressing the possible factors that may influence this standard response to surgery, it is worth examining the mechanism of acute inflammation in more detail.

1.1.2 Acute Inflammation

Acute inflammation is the non-specific response to tissue injury or infection, due to any cause, and is designed to concentrate immune cells at the site of damage. These immune cells act to clear away any necrotic cells and debris, and protect against potential local infection.

The complex series of events that comprises the acute inflammatory response is controlled by chemical mediators, which originate from two main sources (from Roitt *et al.*, 1998):

- **Cellular mediators**
  - Pre-formed *histamine* and s*erotonin* from mast cells.
  - *Prostaglandins & leukotrienes*, originate from cell membrane phospholipids and are produced by a wide variety of leucocytes.
  - *Cytokines*, which are polypeptide products of activated lymphocytes and monocytes and discussed in more detail later.
- **Plasma derived mediators**
  - *Clotting pathway*, which results in the conversion of soluble fibrinogen to insoluble fibrin, and clotting of blood.
  - *Thrombolytic pathway*, generates plasmin which breaks down fibrin and lyses clots.
  - *Kinin system*, which is triggered by activated Hageman Factor XII, and results in the production of the powerful vasodilator bradykinin.
  - *Complement system*, which is a cascade mechanism producing a variety of activated peptides.

The characteristic timing and pattern of production of this plethora of chemical mediators results in the co-ordinated acute inflammatory response. Furthermore, the particular type of response mounted is down to which mediators predominate.

Acute inflammation is initiated by the pre-formed chemical factors, histamine and serotonin, which are released by tissue mast cells, and diffuse towards undamaged blood vessels. The immediacy of the response is enhanced by the fact that no chemical synthesis of these mediators is required. The vascular events that are subsequently triggered are vasodilation and increased permeability, and form the basis of the clinical features of acute inflammation that were first described by Celsus two thousand years ago:

- *Rubor* – The characteristic erythema or redness of inflammation
- *Calor* – The heat associated with inflammation
- *Dolor* – The unmistakeable pain associated with inflammation

The well-described steps of the acute inflammatory response that produce these characteristic manifestations are summarised below (Roitt *et al.*, 1998):

Tissue Damage → Release of preformed factors → Vasodilation and increased permeability → Leucocyte migration into tissues → Phagocytosis of cellular debris

Vasodilation and increased permeability

The combination of vasodilation and increased permeability result in a slowing of blood flow and exudation of protein and fluid into the tissue space that has been damaged. The fluid will dilute any toxins present, and drain any foreign material to the lymphatics, where they can be presented to the immune system. The plasma proteins that initially penetrate to the site of injury are part of the coagulation cascade, and form a fibrin meshwork. This will confine any potential pathogenic agents to the site of tissue damage, and provide a structural framework for the next phase of the inflammatory response – leucocyte migration.

Leucocyte recruitment and migration

Leucocytes can be broadly divided into two main groups, phagocytic cells (*eg* neutrophils, monocytes and macrophages) and lymphocytes (*eg* T-cells and B-cells). The former form the mainstay of the innate immune response, and act to bind, internalise and destroy foreign antigens. The latter are central to the adaptive immune response, co-ordinating and producing a system that retains a memory of previous activity. Acute inflammation forms part of the non-specific innate immune system, and the predominant leucocyte during its early stages is the phagocytic neutrophil. For the neutrophils to perform their function, they must escape from the circulation and gather at the site of tissue damage. This process follows a well described series of events (Simon *et al.*, 2005) – the cells initially come into contact with the endothelium, due to the fluid loss and slowing of blood flow that has previously taken place. They then become tethered to the endothelium, via a series of specific receptors, before adhering strongly and finally passing through intercellular junctions into the interstitium. Once

escaped from the circulation, the neutrophils are attracted to the site of inflammation by a concentration gradient of chemotactic mediators. This is illustrated in Figure 1.1.

Phagocytosis of cellular debris

Having reached the site of tissue damage, as well as performing the phagocytic task of clearing debris, neutrophils will reinforce the initial inflammatory response by producing a number of soluble cytokines, which act to recruit, attract and activate further leucocytes. Cytokines are a large and diverse group of peptides involved in signalling between cells during an immune response. The main types of cytokine are (Roitt *et al.*, 1998):

- **Interleukins (IL-1 to IL-15)**

  Mostly produced by T-cells, but also by other cells. All involved in directing the division and differentiation of other cells.
- **Interferons**

  Involved in limiting and dealing with viral infection.
- **Colony Stimulating Factors**

  Involved in directing the division and differentiation of monocyte precursors in the bone marrow.
- **Others**

  Tumour Necrosis Factors (TNFα and β) and Transforming Growth Factors (TGFβ) – mediate inflammatory and cytotoxic reactions.

All cytokines bind to a specific receptor, activating intracellular signalling pathways that regulate gene transcription.

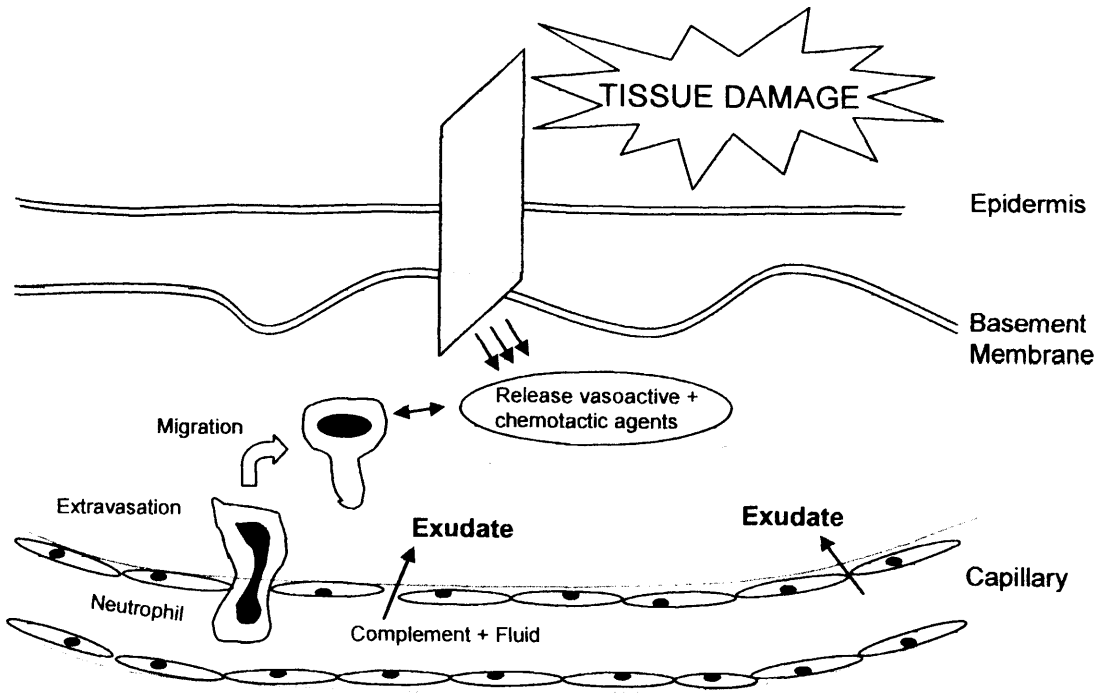


Figure 1.1 Acute inflammatory response to surgery

The trauma of surgery stimulates the release of vasoactive and chemotactic agents, resulting in the formation of an acute inflammatory exudate and the recruitment of neutrophils to the site of tissue damage (adapted from Roitt *et al.*, 1998).

There are several possible eventual outcomes following an acute inflammatory response. Ideally, restitution occurs, with substitution of originally damaged tissue with replacement tissue which is identical in structure and function. This will only occur if the damaged cells possess the capacity to regenerate, which is not often the case. Alternatively, fibrous repair occurs, with production of non-specific scar tissue. Both these scenarios require the elimination of the damaging stimulus. If this does not happen, then a situation of chronic inflammation may develop.

Acute inflammation forms the mainstay of the innate, non-specific immune response to injury. In addition, there exists the adaptive immune response, which possesses the key features of antigen specificity and memory. The link between these two systems is provided by the cells that are recruited to the site of inflammation, and have the ability to phagocytose whole micro-organisms – macrophages and neutrophils. These cells are able to directly recognise invading micro-organisms, using non-specific receptors, and also indirectly, via opsonins such as C3b. Moreover, they can also interact with the T and B-cell lymphocytes that form the basis of the adaptive immune response. B-cells release antibodies, which also act as opsonins, and improve the recognition of pathogens by phagocytes. CD4+ T-helper (Th) cells will only recognise antigens that are presented by "professional" phagocytes which act as "Antigen Presenting Cells". This involves the digestion of a micro-organism and subsequent presentation of a short antigen peptide sequence, within the cleft of a MHC Class II receptor, to that group of Th-cells which recognise it. This clonal expansion of antigen-specific lymphocytes results in the specificity and memory of the response. These Th-cells are then stimulated to release cytokines, which interact with CD8+ T-cytotoxic (Tc) cells and B-cells, and co-ordinate the adaptive immune response. In addition, the Th-cells activate

the antigen-presenting phagocytes, resulting in the destruction of the internalised pathogens.

This, in general terms, is the acute inflammatory response, and a more detailed description can be found in Roitt *et al,* (1998). For the purposes of this thesis, the response to surgery is now discussed in more detail.

1.1.3 Pro-inflammatory Cytokine Response To Surgery

The interest in the immune response to surgery was triggered by the observation that the leading cause of death in surgical patients is multiple organ failure (MOF) (Carrico *et al.,* 1986). A direct relationship was established between the extent of surgical (or traumatic) injury, and the risk of developing sepsis and/or MOF (Baigrie *et al.,* 1992), implicating the immune response as a major factor in outcome.

Surgical trauma induces an acute inflammatory response, as described in Section 1.1.2., the purpose of which is to control existing tissue damage, kill potential infecting organisms and induce the repair processes which lead to a restoration of normal function (Sheeran *et al.,* 1997). The particular pro-inflammatory cytokines which are consequently released, and their functions, are summarised in Table 1.2. Thus, the release of TNFα and Interleukin-1β (IL1β) from macrophages, which have been recruited to the site of tissue damage, has been established as the first phase of the cytokine response to surgery (Baumann *et al.,* 1994; Baigrie *et al.,* 1992). However, the scale of this reaction has been the subject of much debate, with a variety of changes in systemic TNFα and IL1β levels being reported, following major elective surgery (Glaser *et al.,* 1995; Mokart *et al.,* 2002).

Cytokine	Function	Evidence
TNFα	First phase of cytokine response to surgery Activation of coagulation Expression of leucocyte adhesion molecules Release of prostaglandins Induction of muscle catabolism	Baigrie *et al,* (1992) Van der Poll *et al,* (1995)
IL1β	Synergistic role with TNFα Activation of coagulation Release of IL6	Dinarello *et al,* (1991) Baigrie *et al,* (1992)
IL6	Mediator of hepatic acute phase response Release of CRP and α2-macroglobulin Stimulate PBMN proliferation Activator of neutrophils	Gauldie *et al,* (1987) Johnson *et al,* (1998) Schindler *et al,* (1990)

Table 1.2 Key pro-inflammatory cytokines and a summary of their functions

Such discordance is most likely due to differences in the timing of sample-taking, and exacerbated by the relatively short half-lives of these cytokines (*c.* 20 mins) (Bocci, 1991). In the case of the abdominal viscera being the site of damage, macrophage-rich Kuppffer cells may be responsible for a greater degree of this early cytokine release (Enayati *et al.,* 1994). More recently, the measurement of locally-produced cytokines Interleukin-6 (IL6) and TNFα in peritoneal drainage fluid in patients undergoing gastrointestinal surgery revealed significantly higher levels than in the circulation, suggesting a local cause for the systemic response (Jansson *et al.,* 2004; Decker *et al.,* 2005).

TNFα has a multitude of functions during this initial acute phase response, including the activation of coagulation, the expression of leucocyte adhesion molecules, the release of prostaglandins and the induction of muscle catabolism (Van der Poll, 1995). IL1β is the form of IL1 that is more readily detectable in the circulation, and is also primarily released by activated macrophages. It produces much the same result as TNFα (Dinarello, 1991), with such a synergistic role being typical of the cytokine system. One of the key areas of overlap between these two cytokines is in the triggering of the next phase of the inflammatory response, by stimulating the production and release of other cytokines, IL6 being foremost amongst these (Baigrie *et al.,* 1992).

IL6 is a pleotrophic cytokine, and is an important mediator of the hepatic acute phase response to injury and infection. It is constitutively expressed in peripheral blood leucocytes, spleen and liver, and has been found to be inducible in nearly every human tissue and cell type (Biffl *et al.,* 1996). IL6 modulates the local and systemic inflammatory responses, by stimulating the production of further pro-inflammatory

mediators such as C-Reactive Protein (CRP) and α2-macroglobulin (Gauldie *et al.*, 1987; Castell *et al.*, 1989). Such a series of events is supported by e examining the time courses of production of these acute phase proteins: levels gradually rose to a peak at 3-5 days post-surgery, preceded by a rise in serum IL6 (Nishimoto *et al.*, 1989).

As polymorphonuclear cells (PMNs) are one of the key modulators of the initial inflammatory response, their response to IL6 has also been subject to investigation. Like all leucocytes, PMNs are produced from progenitor cells in the bone marrow, and as one might expect, IL6 is a potent stimulator of proliferation. This is evidenced by the observation that mechanical trauma results in an early increase in circulating levels of immature PMNs (Botha *et al.*, 1995). In line with its proinflammatory role, IL6 has been shown to be a potent activator of neutrophils, augmenting their cytotoxic potential by enhancing the release of the protease elastase (Johnson *et al.*, 1998). Furthermore, the level of PMN activation has been correlated with IL6 levels in cholecystectomy patients – those undergoing the more invasive open approach had higher levels of IL6, along with increased PMN superoxide anion release and chemotaxis, compared to laparoscopic patients (Redmond *et al.*, 1994). However, a similar study in children undergoing open or laparoscopic Nissen's fundoplication did not demonstrate any differences between the two groups (McHoney *et al.*, 2005). Nevertheless, overall IL6 seems to play a pivotal role in regulating the immune response.

The serum IL6 response to surgery has been investigated in a number of settings, with a summary of the main studies and their findings included in Table 1.3.

Study Recruits	N°	Main Findings	Ref.
Open cholecystectomy patients	26	- Rise in plasma IL6 levels within 90mins of incision - Levels peak at 4-6hrs - Levels rose in proportion to duration of surgery	Shenkin *et al,* (1989)
Elective AAA and hernia patients	25	- Rise in plasma IL6 levels, peaking at 4-48hrs - Elevated levels still present at 72hrs - Greater rises in AAA compared to hernia patients	Baigrie *et al,* (1992)
Wide variety of surgical patients	28	- Rise in plasma IL6 levels, peaking at 6hrs - Greater rises in cardiac patients, compared to hip replacements	Kragsbjerg *et al,* (1995)
Open colonic resection patients (both Crohn's and carcinomas)	24	- Pre-operatively, Crohn's patients had higher IL6 levels than those with malignancy - Post-operative rise in plasma IL6 levels, prolonged in those with septic complications	Riche *et al,* (1995)
Laparoscopic and open cholecystectomy patients	28	- Rise in plasma IL6 levels, returning to normal 7 days later - Greater rises in open compared to laparoscopic patients	Bellon *et al,* (1997)
Wide variety of surgical patients	26	- Rise in plasma IL6 levels within hours of surgery - Greater rises in AAA and colorectal cancer surgery compared to hip replacements	Cruickshank *et al,* (1999)
Laparoscopic and open colonic resection patients (both Crohn's and carcinomas)	42	- Rise in plasma IL6 levels, peaking at 6hrs - Greater rises in open compared to laparoscopic patients	Hildebrandt *et al,* (2003)
Laparoscopic and open cholecystectomy patients	41	- Rise in plasma IL6 levels within hours of surgery - Greater rises in open compared to laparoscopic patients - Prolonged rises in those with infectious complications	Schietroma *et al,* (2004)

Table 1.3 Summary of studies investigating the plasma IL6 response to surgery

N°, number of patients in the study population. AAA, abdominal aortic aneurysm.

Thus, a study of elective cholecystectomy patients demonstrated a measureable rise in IL6 levels within 90 minutes after the initial skin incision, peaking at 4-6 hours, the scale of which was proportional to the length of surgery (Shenkin *et al.*, 1989). Similar findings were made with a wider variety of surgical procedures (Cruickshank *et al.*, 1990; Kragsbjerg *et al.*, 1995), with higher levels being found after abdominal aortic surgery (AAA) and colorectal surgery, compared to hip replacements. This led to the added conclusion that post-operative IL6 levels were related to the extent of surgical trauma. Supporting this, IL6 levels have been shown in prospective studies comparing open versus laparoscopic approaches to be proportional to the degree of tissue damage (Bellon *et al.*, 1997; Hildebrandt *et al.*, 2003; Schietroma *et al.*, 2004). Looking at the duration of the IL6 response, it has been found to last up to 72 hours following aortic surgery (Baigrie *et al.*, 1992) but did return to normal pre-operative levels 7 days after cholecystectomy (Bellon *et al.*, 1997).

The extent of the pro-inflammatory cytokine response is likely to be a product of the additive effects of trauma, blood loss, and septic complications (Angele *et al.*, 2002). Thus, the greater levels of such cytokines were found in trauma patients with severe blood loss, compared to patients with trauma alone (Roumen *et al.*, 1993), and in trauma victims with septic complications, circulating levels of TNFα and IL6 were higher compared to those without (Martin *et al.*, 1997). Similarly, the duration of the cytokine response has also been correlated to the severity of insult, in a study comparing mastectomy *vs* gastrectomy (Shirakawa *et al.*, 1998). Related studies measured a variety of other cytokines following a surgical or traumatic insult, and characterised a later but prolonged rise in the anti-inflammatory Interleukin-10 (IL10) (Mokart *et al.*, 2002) and TGFβ (Miller-Graziano *et al.*, 1991) – as illustrated in Figure 1.2.

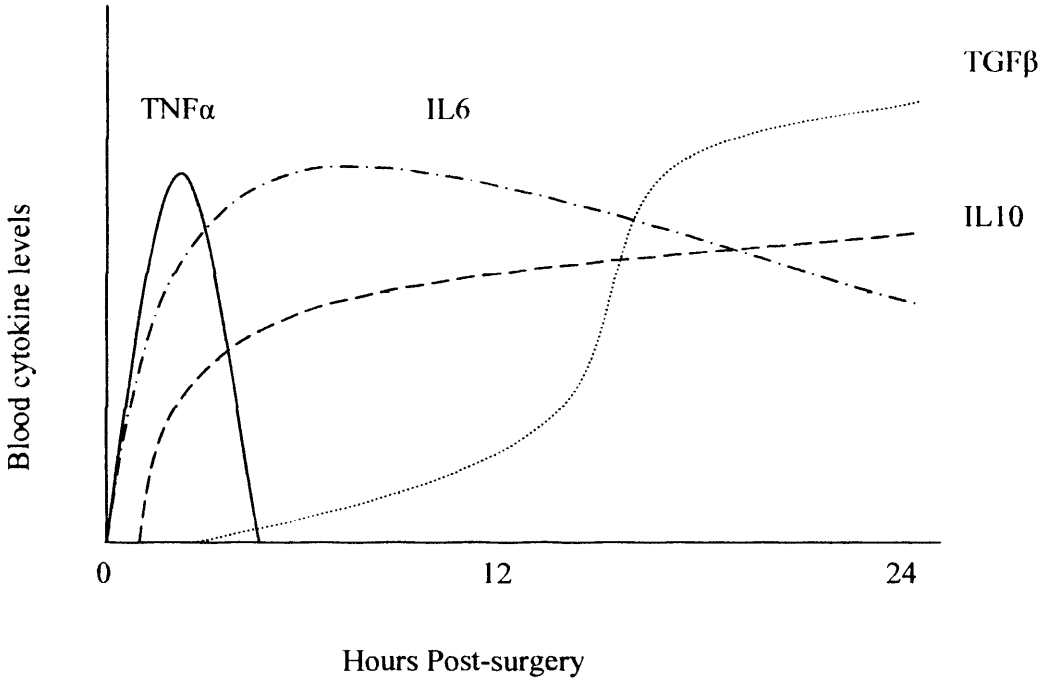


Figure 1.2 Cytokine responses to surgery

The trauma of surgery stimulates a characteristic pattern of cytokine release. Initially pro-inflammatory TNF and IL6 predominate with later anti-inflammatory TGFβ and IL10 (adapted from Angele *et al.*, 2002).

Thus the concept became established of an initial systemic inflammatory response to injury (SIRS), followed by a period of convalescence, mediated by a counter-regulatory anti-inflammatory response (CARS) (Bone, 1996), which is illustrated in Figure 1.3.

1.1.4 Immunosuppressive Cytokine Response To Surgery

The well-described pro-inflammatory initial response to surgery does not explain the apparent impaired immunity, with consequent increased susceptibility to infection and sepsis, observed in the early post-operative period (Hensler *et al.*, 1997). Such apparent immune dysfunction would be in keeping with the CARS hypothesis above – involving a later anti-inflammatory response to surgery. The key cytokines involved in this transition towards a state of immunosuppression are summarised in Table 1.4. IL6 plays a key role in this putative transition, since as well as its pro-inflammatory role it can also act as a powerful anti-inflammatory cytokine, which it achieves by triggering two main mechanisms – the stimulation of specific anti-inflammatory effects, and the down regulation of pro-inflammatory cytokines.

The simplest direct anti-inflammatory effect of IL6 is the inhibition of leucocyte expression of TNFα and IL1β (Schindler *et al.*, 1990). In addition, IL6 stimulates the macrophage production of IL1-receptor antagonist and soluble TNF receptors, which bind IL1 and TNFα, inhibiting them, thereby limiting the inflammatory response (Tilg *et al.*, 1994). In addition to these direct effects, IL6 is also able to act indirectly to limit the inflammatory response. As well as being pro-inflammatory, the IL6-stimulated acute phase response also enhances the synthesis of endogenous glucocorticoids (Chrousos, 1995) and induces the production of prostaglandin E_2 (PGE_2) by macrophages. Glucocorticoids possess well-established anti-inflammatory properties,

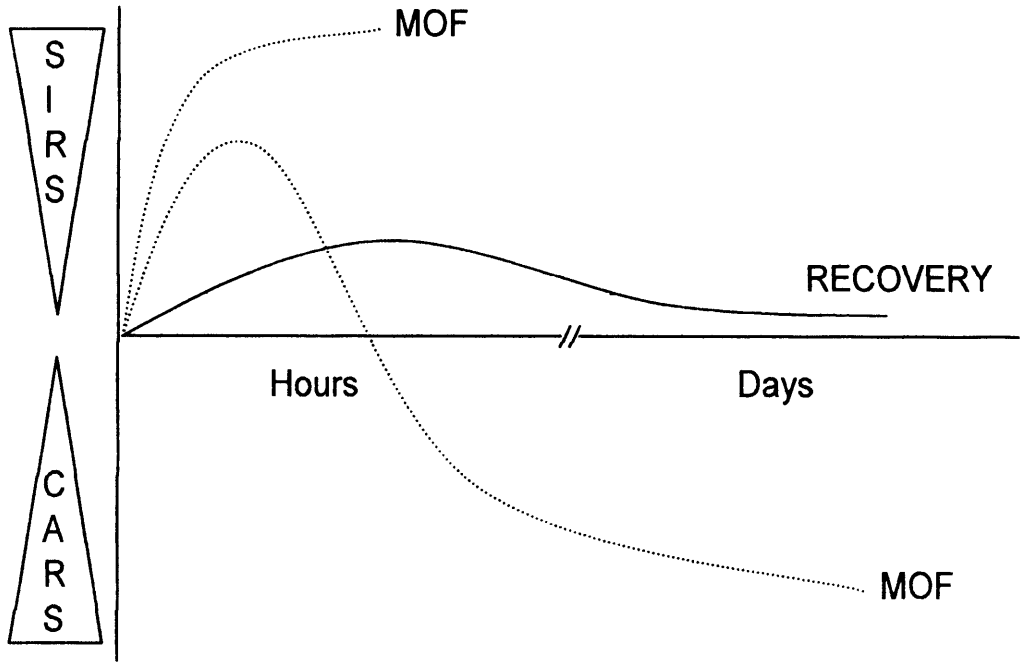


Figure 1.3 Schematic of inflammatory response to surgery

MOF, multi-organ failure. The trauma of surgery stimulates a systemic inflammatory response (SIRS) followed by a period of convalescence mediated by the counter-regulatory anti-inflammatory response (CARS). Excessive SIRS or CARS responses can both lead to deleterious effects (broken lines), whereas normal recovery requires a period of SIRS followed by a return to homeostasis. (Adapted from Lin *et al.*, 2000).

Cytokine	Function	Evidence
IL6	Stimulator of later anti-inflammatory response	Tilg *et al,* (1994)
	Inhibitor of leucocyte release of TNFα & IL1β	Chrousos, (1995)
	Production of soluble TNFα & IL1β receptors	Ayala *et al,* (1994)
	Production of glucocorticoids & prostaglandins	
	Release of IL10 (via PGE_2)	
IL10	Deactivation of monocytes, impairing antigen presentation	Klava *et al,* (1997)
	Inhibits production of IFNγ, IL6 and TNFα	Roitt *et al,* (1998)
TGFβ	Deactivation of monocytes, impairing antigen presentation	Ayala *et al,* (1993)
	Impairs almost all immune functions	Roitt *et al,* (1998)

Table 1.4 Key anti-inflammatory cytokines and a summary of their functions

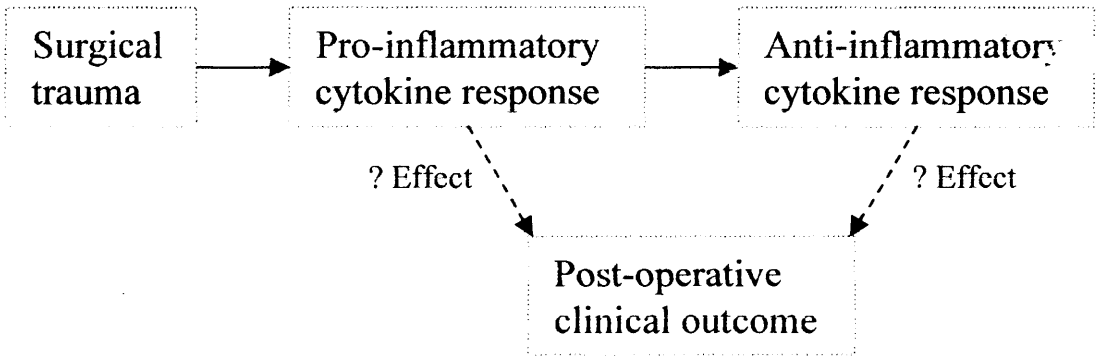
and have been shown to attenuate the systemic inflammatory response in healthy subjects, by altering the pattern of cytokine expression (Barber *et al.*, 1993). More specifically, glucocorticoids induce the anti-inflammatory IL10 (Van der Poll *et al.*, 1996), decrease TNFα and IL1 transcription, reduce generation of inducible cyclo-oxygenase-2 and diminish leucocyte adhesion molecule expression (Scumpia *et al.*, 2005). PGE_2 acts as another powerful endogenous immune suppressant. It also stimulates macrophage production of IL10, via IL4 (Ayala *et al.*, 1994) and decreases synthesis of the pro-inflammatory TNFα and IL1β, via intracellular elevation of the second messenger cAMP (Phipps *et al.*, 1991).

As well as IL6 and IL10, TGFβ acts as an anti-inflammatory cytokine, with levels becoming detectable roughly 24 hours following surgical trauma, and persisting until 72 hours (Hafez *et al.*, 2000). Both IL10 and TGFβ are able to deactivate monocytes, by impairing their antigen-presenting ability (Klava *et al.*, 1997; Ayala *et al.*, 1993).

Turning attention to the adaptive immune response, the traumatic insult of surgery has also been associated with a paralysis of cell-mediated immunity, resulting in the development of sepsis (Levy *et al.*, 1984). Looking at this phenomenon in more detail, involves closer analysis of the effects of surgery on the co-ordinating cells of this response, the CD4+ T-helper (Th) cells. Th-cells can be subdivided into two functionally distinct groups, based on their pattern of cytokine release and subsequent function. Th1 cells are pro-inflammatory, releasing IL2, IL12 & Interferon gamma (IFNγ), whereas the predominantly anti-inflammatory Th2 cells release IL4, IL5, IL6 & IL10 (Angele *et al.*, 2002).

Following surgery, the balance of Th1:Th2 cells shifts towards Th2 predominance (Hensler *et al.*, 1997), with a resulting anti-inflammatory cytokine profile and consequent immunosuppression. Interestingly, this effect is lessened when comparing endovascular to open abdominal aneurysm repair (Decker *et al.*, 2003), and laparoscopic with conventional colorectal resection (Ordemann *et al.*, 2001). Furthermore, it is completely eliminated when the degree of surgical trauma is minimised by using a laparoscopic approach for cholecystectomy (Brune *et al.*, 1999).

In conclusion, as illustrated schematically below, surgery results in an initial acute inflammatory response followed by a period of immunosuppression, affecting both the innate and adaptive immune systems. This is evidenced by the characteristic pattern of cytokine release observed in post-operative patients. Although this is of academic interest, it is the consequences of this surgical trauma-induced inflammation and immunosuppression that is of practical value, and is addressed in the following section.



1.1.5 Inflammation and Surgical Outcome

The extent of the early inflammatory response to surgery may be a determinant of clinical outcome. It is important to note that it is not only the extent of surgical trauma which will influence the scale of post-operative inflammation; choice of anaesthesia, drugs, blood transfusion and visceral ischaemia will also all be modulating factors (Angele *et al.*, 2005). Nevertheless, regardless of the cause, patients with a greater pro-inflammatory (IL1β and TNFα) cytokine response to major surgery have been found to be at greater risk of adult respiratory distress syndrome (ARDS), multiple organ failure (MOF) and mortality (Roumen *et al.*, 1993). As included in Table 1.3, a number of studies have also demonstrated a significant increase in IL6 following AAA surgery, correlating with the degree of surgical trauma (Baigrie *et al.*, 1992; Cruickshank *et al.*, 1999), but in none of these, was this finding a predictor of clinical outcome (Swartbol *et al.*, 2001).

However, such an association between the cytokine response to surgery and the development of post-operative complications has been shown in numerous studies, as summarised in Table 1.5. In particular, an elevated IL6 response in poorer outcome patients has been reported following coronary artery bypass grafting (CABG) (Gaudino et al 2003), emergency AAA (Bown *et al.*, 2004), cholecystectomy (Schietroma *et al.*, 2004) and major oncological surgery (Mokart *et al.*, 2005). Of note, this has not been replicated in colorectal cancer patients (Miki *et al.*, 2005; Sarbinowsi *et al.*, 2005). As well as systemic cytokine response, local peritoneal release in laparotomy patients has also been studied; intra-abdominal septic complications have been found to be accompanied or preceded by a rise in peritoneal TNFα (van Berge Henegouwen *et al.*, 1998).

Study Recruits	N°	Main Findings	Ref.
Elective AAA and hernia patients	25	- Exaggerated and early rise in plasma IL6 levels was associated with development of subsequent major complications	Baigrie *et al*, (1992)
Major trauma, elective and emergency AAA patients	66	- Patients developing ARDS, MOF and dying had elevated IL1β and TNFα at 6hrs compared to uncomplicated recoverers	Roumen *et al*, (1993)
Open colonic resection patients (both Crohn's and carcinomas)	24	- Post-operative rise in plasma IL6 levels, prolonged in those with septic complications	Riche *et al*, (1995)
Elective CABG patients	110	- Patients developing post-operative atrial fibrillation had elevated levels of IL6 following surgery	Gaudino *et al*, (1995)
Elective major oncological resection patients	30	- Post-operative increase in IL6 associated with septic morbidity - Post-operative increase in IL1 receptor antagonist associated with septic shock	Mokart *et al*, (2002)
Elective CABG patients	111	- Correlation found between elevated post-operative IL6 levels, and the development of renal and pulmonary complications	Gaudino *et al*, (2003)
Elective CABG patients	110	- Higher post-operative IL10 levels were associated with subsequent organ dysfunction	Galley *et al*, (2003)
Elective and emergency AAA patients	135	- In elective patients, higher post-operative IL10 levels were associated with prolonged critical care and total hospital stay - In emergency patients, higher levels of IL6 and TNFα found in those developing MOF	Bown *et al*, (2004)
Laparoscopic and open cholecystectomy patients	41	- Prolonged post-operative rises in IL6 in those who subsequently developed infectious complications	Schietroma *et al*, (2004)
Elective major oncological resection patients	50	- IL6 levels at 24hrs post-operation were predictive of subsequent sepsis	Mokart *et al*, (2005)

Table 1.5 Summary of studies investigating plasma cytokine response to surgery and relation to outcome

N°, number of patients in study group. AAA, abdominal aortic aneurysm repair. ARDS, adult respiratory distress syndrome. MOF, multiple organ failure. CABG, coronary artery bypass grafting.

The immunosuppression following major trauma and haemorrhagic shock has been well described, and correlated with high morbidity and mortality (Ertel *et al.*, 1995). The situation for elective surgery is less clear, probably because the expected complications are less pronounced (Menger *et al.*, 2004). As with cases of an overwhelming excessive inflammatory response, it is a loss of normal cytokine balance that is likely to be responsible for any consequences. An early study of cytokine response to elective aortic surgery found an increase in IL6 and IL1β levels, with the former being related to the severity of trauma, and potentially predictive of major complications (Baigrie *et al.*, 1992).

In patients undergoing major surgery for cancer (both gastro-intestinal and gynaecological), post-operative increases in IL6 were associated with septic morbidity and raised levels of IL1 receptor antagonist (another anti-inflammatory cytokine) predicted post-operative septic shock (Mokart *et al.*, 2002). A subsequent study of a similar surgical patient population by the same group concluded that the addition of a clinical assessment of SIRS at Day 1 allowed the rise in IL6 levels to be even more sensitive and specific in the prediction of post-operative sepsis (Mokart *et al.*, 2005). In addition, increased serum IL10 levels following CABG have been correlated to poorer post-operative outcome in adults, as defined by evidence of organ dysfunction (Galley *et al.*, 2003). Similarly, high levels of IL10 following elective AAA repair has been associated with prolonged critical care and hospital stay (Bown *et al.*, 2004).

Rather than looking at the systemic cytokine levels, other studies have focused on the Th1/Th2 cell balance, as described earlier. One study of patients undergoing curative surgery for gastro-intestinal malignancy found that suppression of Th1 cell and

monocyte function in the early post-operative period was directly related to the occurrence of septic complications (Tatsumi *et al.*, 2003).

In summary, a rise in both pro- and anti-inflammatory cytokines has been documented in elective surgical patients, and the pattern and scale of these have been correlated with outcome, as illustrated in Figure 1.4. Much work has focussed on IL6, as it has both pro- and anti-inflammatory activity, and provides a link between these two phases of post-operative recovery. However, most of this has concentrated on generic responses to surgery, yet individuals will differ in the extent of their reaction to surgery. The basis of such individual variation, and its potential importance in influencing surgical outcome, will be addressed in the next section.

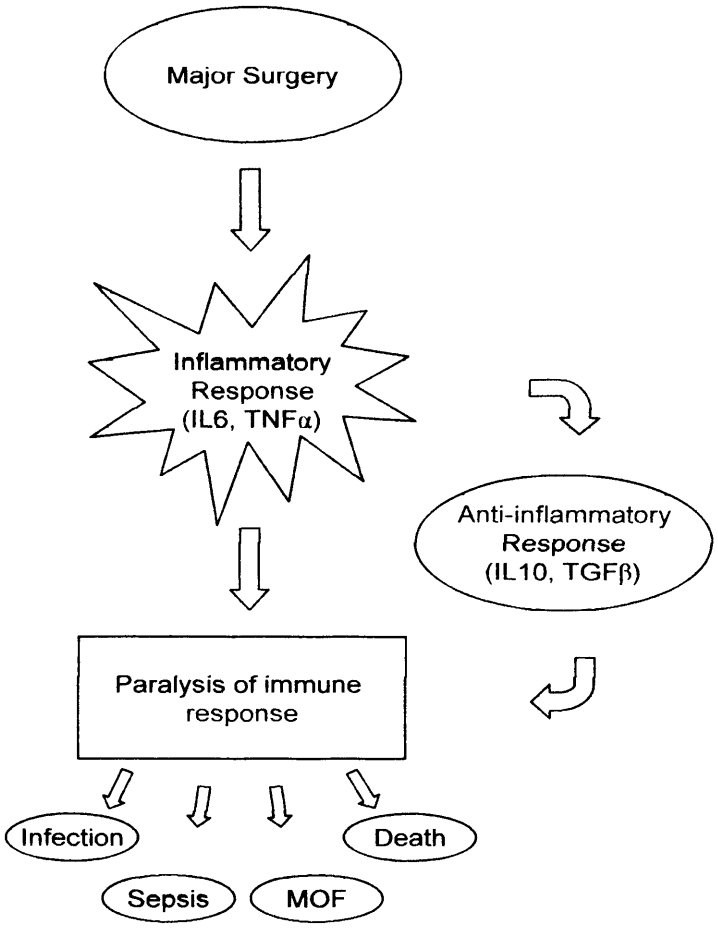


Figure 1.4 Inflammatory responses to surgery and outcome

MOF, multiple organ failure. The inflammatory response to the trauma of surgery can lead to paralysis of the immune response, either directly, or via a subsequent anti-inflammatory response. Poor outcome will follow.

1.2 GENETICS AND SURGICAL OUTCOME

1.2.1 Background

A recent meta-analysis suggests that genetic variants influence an individual's susceptibility to disease (Lohmueller *et al.*, 2003). The outcome from surgery is shaped by a combination of a patient's ability to tolerate the procedure, and their response to it, both in turn influenced to some degree by genotype. Level of fitness is one of the major factors determining the ability to cope with the insult of surgery (Gerson *et al.*, 1990). Furthermore, twin studies have shown genetic causes to be responsible for approximately 40% of the differences in fitness between individuals (Bouchard *et al.*, 1986). Similarly, as has been described in Section 1.1, the inflammatory response to the insult of surgery will also influence outcome, and as will be discussed in Section 1.2.2, a genetic basis is likely to be responsible for the observed inter-individual variation in this reaction. It is thus reasonable to suppose that, as well as influencing susceptibility to disease, genetic makeup may also affect the outcome from surgery.

This is of more than simple academic interest. Despite the ethical issues raised by genetic testing, identification of genes that regulate the mechanisms of oxygen delivery and inflammatory responses, and thus influence surgical outcome, may allow improved clinical decision making and targeted pre-operative optimisation. In addition, it may lead to outcome improvement via the identification of novel therapeutic targets within the inflammatory and immune cascades. Consequently, investigation of the genetic basis of surgical outcome has begun.

1.2.2 Single Nucleotide Polymorphisms

The human genome is now thought to comprise between some 20,000 to 25,000 genes (coding regions of DNA) inter-dispersed with large regions of non-coding DNA (Human Genome Sequencing Consortium, 2004). In turn, each gene comprises several components. The regulatory promoters and enhancers lie upstream from the gene coding sequence, and control the rate and start site of transcription. The coding sequence is specific to that gene, and determines the order of amino acids that result in the final protein. Each coding sequence is further subdivided into stretches which contain the DNA which will be transcribed, called exons. These are interspersed with sections of "junk" DNA which are spliced out of the original template prior to transcription, and called introns. It is the exons which are linked together and transcribed to form the continuous mRNA sequence, and translated into protein. This well-defined flow of genetic information is termed the central dogma of genetics, and is illustrated in Figure 1.5.

Within the genome a vast number of common, specific variations, or 'polymorphisms', have been identified, affecting a significant proportion of the population. The frequency of these has been defined as >1% of the population, as opposed to mutations which affect <1%, and usually result in disease (Balasubramanian *et al.*, 2004). The specific combination of these per individual determines their particular genotype or genetic makeup. It is important to note that not all these polymorphisms influence gene activity, but those that do are deemed to be "functional" polymorphisms. There have been a wide number of different sequence variations reported as polymorphisms, and some of these are summarised in Table 1.6.

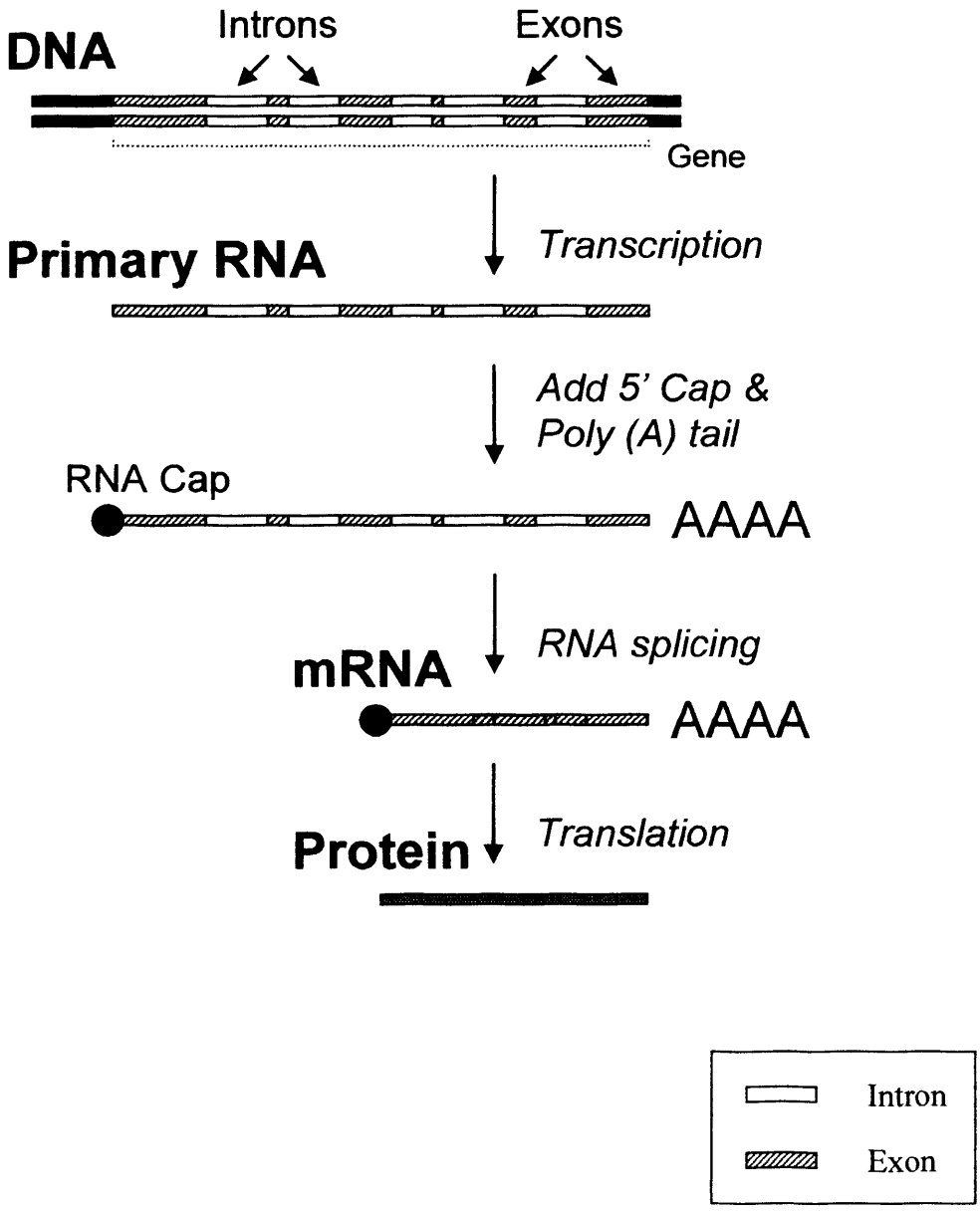


Figure 1.5 Central genetic dogma

5', five-prime end of sequence. The flow of genetic information follows a well-defined path from DNA, through mRNA to protein. The splicing out of introns produces the final continuous mRNA sequence.

Polymorphism Type	Description	Illustration
Single Nucleotide Polymorphism (SNPs)	Substitution of a single base pair in the nucleotide sequence	-GAAT$(CA)_3$TG**T**CA-
Insertion/Deletion Polymorphisms	Insertion or deletion of one or more nucleotides in the sequence	-GAAT$(CA)_3$TG**TTA**CCA-
Variable number of tandem repeats (micro/mini satellites)	Difference in the number of repeat sequences	-GAAT**$(CA)_6$**TGCCA-
		These are variations from the fictitious sequence of:
		-GAAT$(CA)_3$TGCCA-

Table 1.6 Types of genetic polymorphism

The polymorphism changes in the illustrations have been underlined and emboldened. $(CA)_3$ denotes CA repeated three times (*ie* CACACA). Adapted from Balasubramanian *et al,* (2004).

The commonest type of stable genetic variation in the population, occurring in roughly 1 in every 1000 base pairs (Brookes, 1999), involves only a single nucleotide; these are termed single nucleotide polymorphisms (SNPs). Their significance lies with their ability to influence normal gene function, despite the minimal change to the nucleotide sequence and there are a number of mechanisms by which this may occur (Balasubramanian *et al.,* 2004):

- An SNP within the promoter region may affect the binding of transcription factors to DNA, thus altering gene expression.
- An SNP may cause early termination of transcription, resulting in the production of an abnormal protein.
- An SNP at an intron-exon boundary may alter mRNA processing.
- An SNP within the 5' untranslated region of the gene may prevent accurate mRNA translation.

Importantly, genes often contain more than one common SNP, and the specific combination of these present in an individual is termed their haplotype. A specific example of an SNP is the -174 G/C polymorphism, which consists of a substitution of cytosine (C) for a guanidine (G) nucleotide, 174 bases prior to the start-point of transcription of the IL6 gene (Fishman *et al.,* 1998). This SNP will be described in much more detail in Section 1.3, but it is one of the 10% of all SNPs that are functional (Wjst, 2004). The scientific significance of this is that it allows the further investigation of the molecular mechanisms underlying a disease, with the aim of identifying and developing potential novel therapeutic targets. However, even those cases of polymorphisms with no evidence of functionality that have been associated with a

disease, may still prove to be clinically important. For example, if such a non-functional polymorphism proves to be a reliable and consistent marker of disease, then it may potentially be used as an effective screening tool.

1.2.3 Racial differences in Outcome

To investigate, on a global level, whether genotype does influence surgical outcome, one could try to examine the entire genetic code, but this is clearly impractical. However, as different racial groups differ in the pattern of genetic polymorphisms found within their populations (Hoffman *et al.*, 2002), a reasonable compromise is the examination of ethnic differences in outcome. Consequently, a multitude of such studies have been conducted, mostly analysing patients with cancer or vascular disease undergoing surgery. The salient points of the largest of these are summarised in Table 1.7.

Although not a constant finding, racial differences in survival for patients undergoing surgery for cancer have been noted in several retrospective studies (Bradley *et al.*, 2002; Schwarz *et al.*, 2002). Importantly, in patients undergoing surgery for colorectal (Alexander *et al.*, 2004) and prostate (Godley *et al.*, 2003) cancers, this effect was found to be independent of differences in patient demographics, tumour biology, stage of presentation, availability and type of treatment and insurance status. Suggestions for why racial differences in outcome might occur have focused mainly on the possible differences in access to, and standard of surgical care, and in the co-morbidity profiles of different racial groups within the US healthcare system (Godley *et al.*, 2003). Indeed, classifying patients by race, leads to inherent differences in social, environmental, dietary and lifestyle factors, which are extremely difficult to control for and may play a significant role in influencing response to surgery.

Pathology	Study Type	Patient No.	Conclusion	Ref.
All cancers	Retrospective cohort	1.78×10^6	Racial differences in cancer-specific survival and relative risk of cancer death exist, even after controlling for age and stage of disease	Clegg *et al,* (2002)
Cervical cancer	Retrospective cohort	6,050	Race is an independent predictor of survival, after adjustment for age, stage, treatment pattern and other factors	Howell *et al,* (1999)
Breast cancer; male	Retrospective cohort	1,979	Race-dependent differences in survival	O'Malley *et al,* (2002)
Colorectal cancer	Retrospective cohort	3,176	Mortality from all causes was higher among black veterans with colorectal cancer	Dominitz *et al,* (1998)
Colorectal cancer	Retrospective cohort	491	Decreased overall survival at 5 and 10 years post-surgery in African-Americans compared to Caucasians, matched for tumour stage and treatment	Alexander *et al,* (2004)
Gastric cancer	Retrospective cohort	2,043	Asian ethnicity associated with more favourable outcome after curative surgery	Gill *et al,* (2003)
AAA and PVD	Prospective observational	10,355	Race was not an independent predictor of mortality after vascular surgery	Collins *et al,* (2001)
Breast cancer; female <35 yrs	Retrospective cohort	3,978	Poorer overall survival in African-American and Hispanic women after controlling for clinical and demographic characteristics and type of treatment	Shavers *et al,* (2003)
Breast cancer; female	Retrospective cohort	124,934	Differences in stage, treatments and mortality observed by race and ethnicity	Li *et al,* (2003)

Table 1.7 Studies investigating racial and ethnic differences in outcome

AAA, abdominal aortic aneurysm. PVD, peripheral vascular disease.

Nevertheless, suggestions that unknown race-related biological factors may be important have been made (Godley *et al.*, 2003). However, few studies have actually examined genetic differences between patients as the basis for the observed racial differences in outcome. The patient groups that have been investigated are transplant surgery recipients, in particular of the kidney, those undergoing AAA repair, and those having CABG surgery. As inflammatory and immune responses play a significant role following major surgery in determining tissue damage and oxygen demand, and hence outcome, it is relevant therefore that these studies have involved the examination of variations in these responses as a consequence of genetic polymorphism. Such a strategy is supported by the finding that individual variation in cytokine response seen in patients following the same inflammatory stimulus is likely to result from underlying genetic polymorphism (Damas *et al.*, 1997). Consequently, the evidence suggesting a role for genetic polymorphism in determining outcome from surgery is presented in the next four sections.

1.2.4 Organ Transplantation Surgery

More so than any other form of surgery, the potential for rejection means that the success of solid organ transplantation depends on the acute inflammatory and immune responses mounted by the recipient. The magnitude and duration of these are believed to be regulated by the relative levels of pro-inflammatory (for example, IL6, IL2, TNF-α) and anti-inflammatory (IL10, IL4) cytokines (Mytilineos *et al.*, 2004). Consequently, the possibility of developing immunoregulatory therapy that is specifically tailored to an individual's genetic profile has driven the investigation of the genetic basis of this response.

The studies performed to date, investigating the link between a recipient's individual immune response and outcome from transplantation surgery have been extensively reviewed (Marder *et al.*, 2003), with the evidence suggesting a determining role for cytokine genetic polymorphisms. A comprehensive discussion of this work is beyond the scope of this thesis. Nevertheless, briefly, it has been suggested that screening for polymorphisms in TNF-α and IL10 of recipients may help to predict acute graft rejection following kidney transplantation (Sankaran *et al.*, 1999). However, this is not a uniform finding (Mytilineos *et al.*, 2004). Turning to the IL6 gene, possession of the -174 C-allele has been associated with a significantly greater risk of graft loss at three years (Muller-Steinhardt *et al.*, 2002). However, once again this has not been a uniform finding, with other studies failing to show any association between IL6 genotype and renal allograft outcome (Hahn *et al.*, 2001; Hutchings *et al.*, 2002). To complicate the situation further, a role for donor-organ cytokine polymorphisms in influencing a recipient's immune response to renal transplant has also been postulated; donor IL6 CC-genotype has been associated with a significantly increased risk of acute rejection (Marshall *et al.*, 2001).

One of the main reasons for the lack of uniformity amongst all these studies is probably the inherent complexity and redundancy of the inflammatory and immune response. The intricacy of the reaction means that any genetic determining factor is more likely to be polygenic in nature, rather than due to any individual polymorphisms, which inevitably complicates the situation. This is now being taken into account, with a recent study demonstrating a co-operative influence between three separate SNPs within the promoter region of the IL6 gene (including the -174 G/C SNP) on long term kidney allograft survival (Muller-Steinhardt *et al.*, 2004). Other reasons for the equivocal

results include inconsistencies in study design, choice of clinical end-point and baseline disease. There is also a lack of uniformity in the immunosuppression protocols employed between studies, making valid comparison difficult.

Nevertheless, the outcome of transplantation is still likely to be linked to a patient's individual immune response, which is in turn determined by cytokine genetic polymorphisms. However, the specifics of this are yet to be fully established, and it is difficult to generalise from this finding, because of the unique influence of the immune response on allograft success.

1.2.5 Coronary Artery Bypass Grafting Surgery

Another surgical procedure with an exceptional reason to have its outcome determined by the inflammatory response is CABG, with cardiopulmonary bypass. This is due to the use of an intra-operative extracorporeal circulation, which provides a significant and consistent systemic inflammatory stimulus (Butler *et al.*, 1993). The inter-individual differences in surgical outcome observed in these patients have been attributed to the genetic predisposition to developing a major post-operative systemic inflammatory reaction (Roth-Isigkeit *et al.*, 2001). Accordingly, the analysis of specific cytokine gene polymorphisms that may modulate the inflammatory response has received recent attention.

The first gene to be investigated in this way was Apolipoprotein E, which is involved in both acute inflammation and subsequent tissue repair. In the first report of a genetic basis for post-operative outcome, inheritance of the ε4 allele of this polymorphism was associated with a decreased risk of post-operative renal dysfunction following cardiac

surgery (Chew *et al.*, 2000). Since then, this relationship has been confirmed in another study of post-operative CABG patients (MacKensen *et al.*, 2004). A functional polymorphism within the early pro-inflammatory cytokine TNFα has also been investigated. Thus homozygotes for the TNFB2 allele have been shown to have higher post-operative levels of TNFα, and an increased risk of developing left ventricular and pulmonary dysfunction (Tomasdottir *et al.*, 2003). Interestingly, the elevation in TNFα levels correlated with IL6 levels, and unsurprisingly polymorphisms of this key pro-inflammatory cytokine have been of particular interest.

The -174 G/C IL6 polymorphism has been found to significantly affect systemic levels of IL6 following CABG surgery, with the CC homozygotes having a significantly greater rise in IL6 compared to other genotypes (Brull *et al.*, 2001). This functionality has been confirmed in other studies, but in contrast, the GG homozygotes have been found to show a larger rise in post-CABG IL6 levels (Burzotta *et al.*, 2001; Gaudino *et al.*, 2003). These studies also looked at surgical outcome, concluding that GG homozygotes are at greater risk of developing the complications of post-operative atrial fibrillation (Gaudino *et al.*, 2003a), renal and pulmonary dysfunction (Gaudino *et al.*, 2003b) and longer stay in both intensive care (ICU) and hospital overall (Burzotta *et al.*, 2001). As with all of these studies that have investigated the influence of a particular candidate gene on the reaction to surgery, discrepancies may be due to the fact that polymorphisms do not exist in isolation, and combinations or haplotypes are probably of greater relevance. Nevertheless, once again, the ability of the -174 G/C IL6 polymorphism to modulate the inflammatory response to surgery has been demonstrated. Furthermore, the possibility of a genetic modulation of post-operative clinical outcome has been established in this cohort of patients.

1.2.6 Abdominal Aortic Aneurysm Repair Surgery

The final cohort of patients who have been investigated for a potential genetic basis of post-operative outcome are those undergoing AAA surgery. This is due to the extensive nature of the surgery, and in particular, the ischaemia-reperfusion injury caused by cross-clamping of the aorta during the procedure. The overwhelming systemic pro-inflammatory response that is provoked drives the high risk of post-operative MOF. This serious complication is the major cause of peri-operative morbidity and mortality following AAA repair, especially in the cases of rupture (Sayers *et al.*, 1997), and is characterised by the sequential failure of organ systems over time.

Initial studies investigating the nature of inflammatory responses following AAA repair have highlighted a wide variety of cytokine responses to surgery (Roumen *et al.*, 1993; Holmberg *et al.*, 1999), but the most consistent observation has been a variable increase in levels of plasma IL6 (Swartbol *et al.*, 2001). It is thought that the gastrointestinal tract is mostly responsible for this rise in IL6 levels (Norwood *et al.*, 2004). As with the other types of surgical procedure already discussed, the interest in the cytokine response relates to its role in underlying, and potential for predicting, post-operative morbidity and mortality.

Thus, cytokines have been implicated in causing the failure of all the major organ systems. Adult respiratory distress syndrome (ARDS) is an inflammatory pulmonary injury that commonly forms part of MOF post-AAA repair. It is thought to be caused by activated neutrophils, migrating from the pulmonary vasculature into the interstitial spaces, secreting cytokines and producing a local inflammatory response (Mitchell, 1999). These neutrophils are originally activated by the pro-inflammatory cytokine

response to surgery. The impairment of cardiac function following AAA repair is worsened by the increased likelihood of this cohort of patients suffering from ischaemic heart disease, due to the same atherosclerotic pathology underlying both conditions. As such, the diminished cardiac reserve that most AAA patients possess is exacerbated by cytokines such as TNFα, which has been shown to impair cardiac function (Pagani *et al.,* 1992). These pro-inflammatory cytokines will also intensify the ischaemic injury, due to hypotension and supra-renal aortic clamping, suffered by the kidneys during AAA repair. TNFα has been shown to cause direct damage to renal glomeruli (Bertani *et al.,* 1989), as well stimulating their release of further pro-inflammatory mediators (Laufer *et al.,* 1997).

The wide inter-individual differences in response to surgery have been suggested as a cause of the variable cytokine patterns recorded following AAA repair, leading to the investigation of the influence of cytokine polymorphisms. However, this has proved to be a little less conclusive, with an initial study finding significant post-operative rises in plasma IL6, IL10 and TNFα, but failing to demonstrate an association between the magnitude of this response and individual patient cytokine polymorphisms (Bown *et al.,* 2003). Despite this, the same group went on to conduct a prospective study of elective and emergency AAA cases, finding that plasma cytokine levels related to outcome following open AAA repair (Bown *et al.,* 2004). Specifically, elevated post-operative levels of the anti-inflammatory IL10 were associated with prolonged ICU and hospital stay, and high levels of the pro-inflammatory TNF-α and IL6 with mortality and MOF. However, of even greater importance was their conclusion that cytokine gene polymorphisms also show a significant relationship with surgical outcome. Specifically they found the G allele at the IL6 -176 locus to be associated with the development of

post-operative organ failure and A allele at the TNF-α -308 locus with prolonged post-operative ICU requirements after elective AAA repair. So, as with patients undergoing organ transplantation and CABG, the potential for a genetic polymorphism to influence post-operative outcome has been shown in elective AAA repair.

1.2.7 Sepsis

The procedures that have been examined so far are fairly uncommon, and all involve a significantly greater inflammatory response than most operations. For those undergoing more standard and widespread general surgical procedures, initial cytokine responses to the surgical insult are less extreme than in the cases of CABG, AAA repair and transplantation. Rather than MOF due to an overwhelming inflammatory response, post-operative sepsis, possibly due to inadequate inflammatory and immune responses, is a more likely cause of poor outcome. In fact, sepsis is responsible for complications in approximately 10% of patients undergoing a major abdominal operation (Wichmann *et al.*, 2000).

Starting with sepsis from all causes, there is a distinct observed variation in susceptibility and mortality of patients, and genetic polymorphisms may account for this (Lin *et al.*, 2004). For example, looking at preterm infants, who are less likely than adults to have their gene expression influenced by environmental stimuli, the IL6 -174 GG genotype was found to be associated with a >2-fold risk of developing septicaemia (Harding *et al.*, 2003). Similarly, in adult ICU patients, the TNF2 polymorphism of the TNFα gene has been associated with a susceptibility to and mortality from septic shock (Mira *et al.*, 1999).

Moving on to post-operative sepsis, this has also mostly been studied in the ICU setting. In an early study of adult post-surgical ICU, septic patients with a poorer prognosis were found to have elevated plasma levels of TNFα, and tended to have the bi-allelic TNFβ2 *NcoI* polymorphism of the TNFβ gene (Stuber *et al.*, 1996). Similarly, a greater risk of ICU admission, and mortality following the onset of sepsis, has been found in TNFβ2 homozygote post-operative patients (Tang *et al.*, 2000). More recently, in a prospective study of 172 elective patients undergoing major abdominal surgery, TNFβ2 homozygotes had increased risks of all major post-operative complications, and not just those related to sepsis (Riese *et al.*, 2003). A further prospective study of 160 elective patients undergoing major gastro-intestinal surgery suggested that the NcoI TNFβ gene polymorphism influenced development of post-operative complications (Kalhke *et al.*, 2004). The TNFβ2 homozygotes were at greater risk of severe complications, and if they developed post-operative sepsis, then they had a greater mortality. However, this study failed to demonstrate a predictive role for the TNFβ gene polymorphism.

In summary, the finding of a racial disparity in surgical outcome may be partly explained by a difference in genetic polymorphism distribution, and in particular those affecting cytokine genes. Such studies represent the strongest body of evidence suggesting a genotypic influence over post-operative outcome. This hypothesis has only recently begun to be investigated, focusing mostly on surgical procedures known to provoke a particularly large inflammatory response, such as organ transplantation, CABG and AAA repair. To date, only limited conclusions have been reached, but there is evidence to suggest that post-operative morbidity and mortality may be influenced by cytokine polymorphisms.

More specifically, the common -174 G/C polymorphism involving the key pro- and anti-inflammatory cytokine IL6 has emerged as a candidate for such an influential role. The potential importance IL6 lies with its involvement in both the immediate acute inflammatory response, and the transition towards the compensatory anti-inflammatory response. Consequently, this common polymorphism is examined in more detail in the following section.

1.3 INTERLEUKIN 6 -174 G/C SINGLE NUCLEOTIDE POLYMORPHISM

1.3.1 Background

Briefly, IL6 is a highly inducible essential mediator of the acute phase response, which is produced in response to noxious stimuli by a wide variety of cells. It has a short half life, and consequently circulating levels are mostly regulated at the level of transcription (Castell *et al.*, 1988). However, significant inter-individual differences in transcription and expression are observed, which may result from polymorphisms in the promoter region of the IL6 gene (Terry *et al.*, 2000). The clinical significance of this variation is due to the potential role of elevated IL6 production in the pathology of a number of diseases, such as rheumatoid arthritis (Hirano *et al.*, 1988) and systemic-onset juvenile chronic arthritis (S-JCA) (Fishman *et al.*, 1998), conditions with an inflammatory etiology.

In the case of S-JCA, the similarity between one of the cardinal symptoms, the unique pattern of fever, and the acute phase response prompted the investigation of a potential causative role for IL6. Serum IL6 levels were found to spike in concert with fevers (Rooney *et al.*, 1995), leading to the hypothesis that this cyclical pattern may be due to a difference in gene transcription. During the investigation of this, a functional single nucleotide G to C polymorphism at position -174 within the 5' flanking promoter region, upstream from the start site of transcription of the gene, was first described (Fishman *et al.*, 1998). This single nucleotide change was associated with a suppression of inducible IL6 expression; the G-allele associated with greater IL6 production and levels.

Several other functional polymorphisms within the promoter region of the IL6 gene have since been described (Terry *et al.*, 2000), and the main ones are summarised in Table 1.8.

1.3.2 Functional Implications and Association Studies

In the original description of the -174 G/C polymorphism, the C-allele was associated with lower levels of IL6 than the G-allele *in vitro* and in healthy subjects (Fishman *et al.*, 1998). This finding has been replicated in pre-operative patients with colorectal cancer (Belluco *et al.*, 2000). However, there are several problems associated with the use of *in vitro* cell line gene constructs, such as the choice of cell, and the difficulty in modelling the complexity of gene expression regulation *in vivo* (Humphries *et al.*, 2001). So, other studies have found an association between the C-allele and elevated plasma IL6 levels (Jenny *et al.*, 2002; Bruunsgaard *et al.*, 2004).

IL6 and inflammatory stimuli

Given the role and inducible nature of IL6, it is the polymorphic influence over the response to an inflammatory stimulus that is probably of greater consequence. Thus, and in contrast, the stimulated rise in IL6 following CABG surgery has been found to be greater in subjects with CC genotype (Brull *et al.*, 2001). CABG is known to provide a significant, homogeneous and consistent inflammatory stimulus, with cardiopulmonary bypass probably being mostly responsible (Cremer *et al.*, 1996), and so provides an excellent *in vivo* model for studying the IL6 polymorphic effects. Similarly, an association between the C-allele and higher IL6 levels has been reported in patients with an abdominal aortic aneurysm, a condition that is thought to provide a chronic inflammatory stimulus (Jones *et al.*, 2001).

Position	SNP	Effect	Ref.
-597	G→A	- G allele associated with greater transcription in cell line, as part of a haplotype	Terry *et al,* (2000)
-572	G→C	- G allele associated with greater transcription in cell line, as part of a haplotype	Terry *et al,* (2000)
-373	AnTn	- 8A/12T allele associated with decreased transcription in cell line, as part of a haplotype	Terry *et al,* (2000)
-174	G→C	- C allele associated with decreased plasma levels in patients	Fishman *et al,* (1998)
+1753	C→G	- Both C & G alleles associated with poorer outcome in critically ill adults, as part of a haplotype	Sutherland *et al,* (2005)
+2954	G→C	- Both C & G alleles associated with poorer outcome in critically ill adults, as part of a haplotype	Sutherland *et al,* (2005)

Table 1.8 Summary of key functional IL6 gene SNPs

The position relates to the start site of transcription. AnTn, a run of nucleotides of varying length. The haplotype refers to specific combinations of SNPs, as described in the appropriate references.

So, given the established functional role of the G/C polymorphism in determining IL6 basal levels and response to a noxious stimulus, it is unsurprising that its relevance has been investigated in a number of inflammatory conditions. The original description of the polymorphism was in S-JCA, with this study concluding that the CC genotype was significantly less common in affected patients, and may exert some form of "protective" effect (Fishman *et al.*, 1998).

Septicaemia

Septicaemia, another inflammatory condition involving documented infection within the circulation, has also been investigated. Other pro-inflammatory cytokine genetic polymorphisms have been shown to contribute to outcome from septicaemia, such as TNFα (Mira *et al.*, 1999) and IL1 (Read *et al.*, 2000), making it a reasonable hypothesis that the -174 G/C IL6 polymorphism is of relevance. In a study of pre-term infants with septicaemia, lower basal levels of IL6 were associated with greater vulnerability, and the GG genotype with increased risk (Harding *et al.*, 2003). However, a similar study of adults in intensive care with septicaemia failed to show any genotypic association with incidence, although, somewhat surprisingly, mortality was less in patients with the GG genotype (Schluter *et al.*, 2002). The difference between adult and neonatal findings probably relates to the inevitably greater gene-environment interaction in the former group. This is supported by the finding of close regulation of IL6 production in neonates, but not in adults (Kilpinen *et al.*, 2001). Probably of greater importance, is the fact that IL6 has more than one functional polymorphism, and the combination of these (*ie* haplotype), rather than any single SNP, may be significant. Consequently, an investigation of IL6 and outcome in critically ill adults with systemic inflammatory response syndrome, demonstrated a haplotype association with organ dysfunction and

mortality, but no influence for the G/C polymorphism in isolation (Sutherland *et al.*, 2005). Hence, the association between IL6 genotype and outcome in septic patients remains unclear. The situation is only slightly clearer in another chronic inflammatory condition – cancer.

Cancer

It seems well established that high IL6 levels suggest an advanced stage and poorer outcome for a number of tumour types, including prostate (Shariat *et al.*, 2001), oesophagus (Oka *et al.*, 1996), stomach (Wu *et al.*, 1996) and colon (Belluco *et al.*, 2000). This forms the basis for the putative association between the functional G/C polymorphism, incidence and outcome in cancer patients. The CC-genotype has been associated with increased risk of bladder (Leibovici *et al.*, 2005), colorectal (Landi *et al.*, 2003) and breast cancer (Hefler *et al.*, 2005), and interestingly, the C-allele has been linked to a more aggressive breast cancer phenotype (Iacopetta *et al.*, 2004). However, the C-allele has also been associated with low stage in ovarian cancer (Hefler *et al.*, 2003), although such discrepancies in findings may well be due to differences in tumour-type. The association with cancer outcome is also unclear. Unexpectedly, despite the above, the C-allele has been linked to improved disease-free and overall survival in breast (DeMichele *et al.*, 2003) and ovarian cancer (Hefler *et al.*, 2003). On the other hand, the C-allele correlates with increased risk of advanced and aggressive disease, and recurrence, in prostate cancer patients (Tan *et al.*, 2005).

Cardiovascular disease

As well as cancer, the other main condition that has been examined for association with IL6 genotype is cardiovascular disease, stemming from the mounting evidence that

inflammation is a risk factor for coronary artery disease. For example, chronic inflammation, due to cigarette smoking, is linked to increased risk (de Maat *et al.*, 1996), whereas lower baseline levels of inflammatory markers, due to moderate physical fitness decreases the risk (Cooper *et al.*, 1976). Furthermore, the degree of inflammation also correlates with the severity of cardiovascular disease (Heinrich *et al.*, 1995). In fact, plasma IL6 levels have been reported as a marker for sub-clinical cardiovascular disease (Jenny *et al.*, 2002). It is possible therefore that IL6 and its functional G/C polymorphism can both influence the risk of cardiovascular disease (Woods *et al.*, 2000).

This premise has been investigated in a variety of populations. In healthy men, the C-allele was linked to significantly higher systolic blood pressure and risk of a coronary heart disease event, particularly in smokers (Humphries *et al.*, 2001). However, in a more comprehensive study population of elderly men and women, IL6 genotype did not appear to directly influence the risk of cardiovascular events, although the suggestion was made that the C-allele contributes to the development and progression of atherosclerotic disease (Jenny *et al.*, 2002). A subsequent study of 80 year old subjects also concluded that the C-allele associated with prevalence of cardiovascular disease, and increased mortality in smokers (Bruunsgaard *et al.*, 2004). Looking at a possible mechanism for these observations, a study of healthy volunteers by Brull and co-workers (2002) demonstrated an association between endothelial dysfunction and the C-allele in men; an effect amplified by smoking.

Overall, the common functional -174 G/C polymorphism is able to influence levels of IL6. Given the central role played by this cytokine in the acute phase response, much

work has focused on the association between this polymorphism and the incidence and outcome of a multitude of inflammation-mediated conditions, including critical illness, cancer and cardiovascular disease, and the findings of some of these key functional association studies are summarised in Table 1.9. However, as described in Section 1.1.3, IL6 is only one of many pro-inflammatory cytokines, and others such as TNFα also have common functional polymorphisms that have been similarly investigated. Moreover, it is becoming increasingly evident that another is angiotensin converting enzyme, and the relevance of this molecule is addressed in the following section.

Study Recruits	N°	Main Findings	Ref.
Pre-term infants with septicaemia	157	- GG genotype associated with lower basal IL6 production and increased risk of development of septicaemia	Harding *et al*, (1992)
Critically ill adults in ICU	326	- GG genotype associated with better outcome in those developing septicaemia, but no evidence of influence on plasma IL6 levels	Schluter *et al*, (2002)
Caucasian critically ill adults in ICU	228	- Haplotype combinations of 3 SNPs were associated with increased mortality and organ dysfunction, rather than any individual -174 G/C effect	Sutherland *et al*, (2005)
Middle-aged healthy UK men	2751	- C allele associated with higher systolic blood pressure	Humphries *et al*, (2001)
Small (≤5cms) AAA patients	466	- C allele associated with increased risk of cardiovascular mortality	Jones *et al*, (2001)
Elderly adults	5888	- C allele associated with increased risk of myocardial infacts	Jenny *et al*, (2002)
80 year old adults	324	- Increased prevalence of cardiovascular disease in CC homozygotes	Bruunsgard *et al*, (2004)
Surgical patients operated on for breast cancer	256	- CC homozygotes were more likely to have high grade tumours and had worse overall survival	Iacopetta *et al*, (2003)
Female breast cancer patients with ≥4 positive axillary nodes	80	- GG homozygotes had worse disease free and overall survival times compared to C-allele carriers	DeMichele *et al*, (2005)

Table 1.9 Summary of key association studies investigating potential functional influence of the IL6 -174 G/C polymorphism

N°, number of patients. ICU, intensive care unit. SNP, single nucleotide polymorphism. AAA, abdominal aortic aneurysm.

1.4 ACE AND INFLAMMATION

1.4.1 Angiotensin Converting Enzyme

Angiotensin Converting Enzyme (ACE) is one of the key parts of the Renin-Angiotensin System (RAS). The RAS exists at both a systemic level, which regulates blood pressure and fluid homeostasis, and also a local tissue level, where its significance probably lies with growth stimulation, but is still subject to much debate (Lavoie *et al.,* 2003). In either case, the basic substrate of this cascade is angiotensinogen, a circulating α_2-globulin, synthesized in the liver. This is cleaved to form the decapeptide angiotensin I (Ang-I) by renin, a proteolytic enzyme which is produced and stored in the juxta-glomerular cells surrounding the afferent arterioles within the glomeruli of the kidney. Ang-I is in turn cleaved by ACE to produce the major effector peptide of the RAS, angiotensin II (Ang-II). ACE is classically present in the lung and endothelial cells, but has been described in a multitude of tissues, including heart, kidney, leucocytes and peripheral monocytes (Igic *et al.,* 2003). However, the major site of Ang-II production is thought to be the vasculature (Watanabe *et al.,* 2005). Further degradation of Ang-II can occur, forming the less active heptapeptide Ang-III. The RAS is summarised in Figure 1.6.

The actions of Ang-II result from its binding to specific receptors AT-1 and AT-2, which are classified according to their differential affinities for various antagonists (Timmermans *et al.,* 1993). These are G protein-coupled receptors, and are found on cell surfaces. Both are present in a number of tissues, in particular kidney, brain and adrenal gland, but most of the physiological actions of the Ang-II are mediated by the AT-1 (Timmermans *et al.,* 1993).

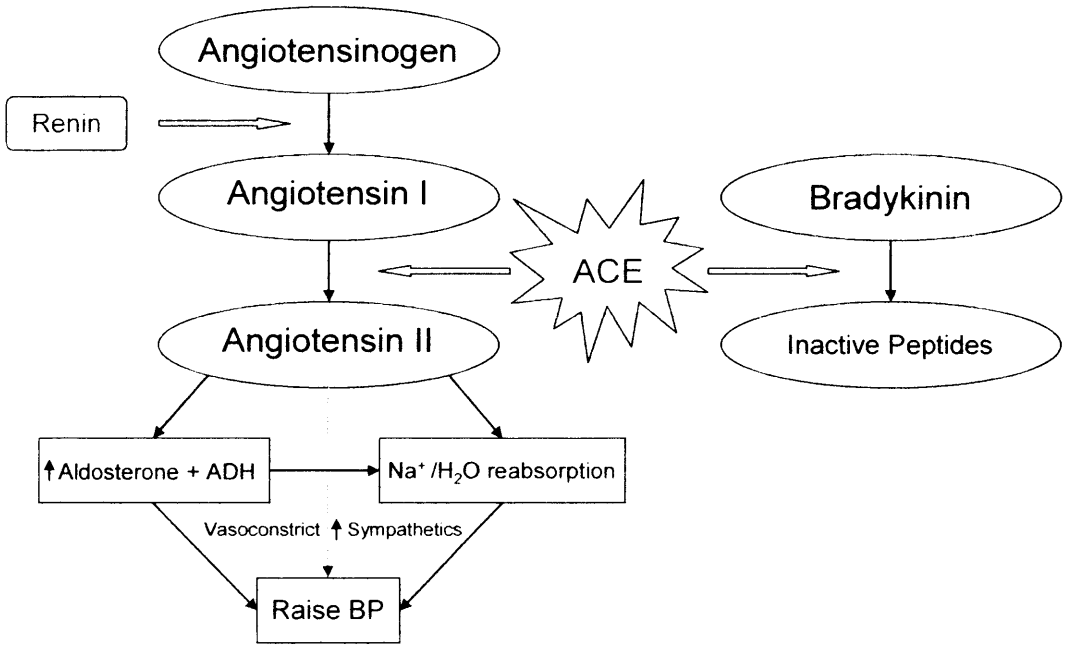


Figure 1.6 Schematic of the renin-angiotensin system

ACE, angiotensin converting enzyme. ADH, antidiuretic hormone. BP, blood pressure. ACE catalyses both the production of Angiotensin II and the breakdown of Bradykinin into inactive peptides.

The exact role of the AT-2 receptor remains unclear, but an opposing role against AT-1 has been suggested (Carey *et al.*, 2000). A vasodilation-mediating AT-4 receptor has also been described, which is not G protein-coupled and binds one of the breakdown products of Ang-II (Patel *et al.*, 1998). However, its significance in humans remains unclear.

Systemic Ang-II is one of the key modulators of blood pressure, acting to protect circulatory homeostasis via a number of mechanisms. In the immediate aftermath of a hypotensive crisis, such as a major operation with significant bleeding, Ang-II will aim to maintain blood pressure by causing vasoconstriction both directly, by stimulating receptors present in the vasculature, and indirectly, by increasing sympathetic tone. In addition, Ang-II will degrade the peptide bradykinin, which normally produces vasodilation via its Type II (Bk_2) receptor. More chronically, Ang-II can restore fluid balance in a number of ways (Weir *et al.*, 1999):

- Direct action on renal AT-1 receptors, increasing sodium and thus water reabsorption
- Indirect renal action, via the stimulation of production of aldosterone from the adrenal cortex, which enhances water reabsorption from the collecting ducts
- Indirect central action, via the stimulation of the sensation of thirst

This ability of the RAS to modulate systemic blood pressure formed the rationale for the development of ACE inhibitor and AT receptor antagonist therapies, which have been shown to be of benefit in patients at high risk of cardiovascular events (Yusuf *et al.*, 2000). However, two interesting study findings point to the local tissue RAS, as

well as the systemic RAS, being an important therapeutic target. Firstly, the anti-hypertensive actions of ACE inhibitors correlate better with the inhibition of tissue ACE, rather than plasma ACE. Furthermore, patients with raised blood pressure, but normal or low plasma levels of ACE can still be effectively treated with ACE inhibitors (Dzau *et al.*, 2001).

Quite what the role played by the tissue RAS is remains unclear. In the kidney, it is thought to regulate local blood flow, and sodium reabsorption (Navar *et al.*, 1997). In the brain, it may be involved in neurotransmission and sympathetic outflow (Steckelings *et al.*, 1992). In the vasculature, local RAS has been implicated in smooth muscle cell growth (Girendling *et al.*, 1994). However, the study findings that pointed to the importance of the local tissue RAS, may in fact be suggesting that ACE inhibitors possess other desirable properties, other than the ability to block the formation of ACE. Thus the hypothesis that ACE inhibitors are also anti-inflammatory, making both ACE and Ang-II pro-inflammatory molecules, has been made (Das, 2005).

1.4.2 ACE as a Pro-inflammtory Cytokine

Atherosclerosis is the common denominator in the majority of cardiovascular diseases, for which ACE inhibition or AT receptor blockade has been shown to be of benefit. It is also becoming clearer that atherosclerosis involves chronic inflammation (Libby, 2006). Thus, in an attempt to understand the favourable effects of pharmaceutically manipulating the RAS, some studies have examined their influence on systemic markers of inflammation. AT receptor blockade has been shown to cause a fall in levels of the acute phase CRP in both normal subjects (Dandona *et al.*, 2003) and those treated for essential hypertension (Dohi *et al.*, 2003). Similarly, both AT blockade and ACE

inhibitors decrease plasma levels of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which are involved in leucocyte extravasation (Graninger *et al.,* 2003). Hence, the findings of these clinical studies support the suggestion that ACE inhibitors and AT receptor blockade are able to suppress inflammation. To investigate this further on a molecular level, the effects of the RAS on the three main steps of the acute inflammatory response (described in Section 1.1.2) are examined in turn.

1. Increase in vascular permeability

The initial phase is a local increase in vascular permeability, resulting in the formation of the acute inflammatory exudate. Two of the main mediators that result in this phenomenon are prostaglandins (Yoshikai, 2001) and vascular endothelial growth factor (VEGF) (Dvorak *et al.,* 1995), and Ang-II is able to induce the production of both of these, as well as increasing permeability by pressure-mediated mechanical injury to the endothelium (Cheng *et al.,* 2005). In particular, the relationship between Ang-II and VEGF has been the subject of much investigation. Starting with experimental evidence, Ang-II has been found to stimulate the synthesis and secretion of VEGF in human mesangial cells (Pupilli *et al.,* 1999). Furthermore, a synergistic relationship between the RAS and VEGF has been found, with VEGF being able to induce ACE in human umbilical endothelial cells (Saijonmaa *et al.,* 2001). Clinically, this interaction between ACE and VEGF has been demonstrated in conditions involving increased vascular permeability - proliferative diabetic retinopathy (Wilkinson-Berka, 2001) and ovarian hyperstimulation (Elchalal *et al.,* 1997).

2. Leucocyte recruitment and accumulation

The next phase of inflammation involves the recruitment, extravasation and accumulation of leucocytes. The initial recruitment requires an increase in the expression of adhesion molecules on both leucocytes and endothelial cells. Ang-II has been shown to achieve this in both endothelial cell models (Kim *et al.*, 1996) and *in vivo* in rats (Alvarez *et al.*, 2004). More specifically, P- and E-selectins, which are expressed by endothelial cells and aid the capture of leucocytes, are upregulated by Ang-II (Grafe *et al.*, 1997; Tayeh *et al.*, 1998). The expression of endothelial ICAM-1 and VCAM-1, along with integrins on leucocyte surfaces, is also upregulated by Ang-II in rat models (Tummala *et al.*, 1999; Alvarez *et al.*, 2004). Clinical supporting evidence lies with the analysis of patients, such as those with chronic heart failure, who have raised soluble levels of these adhesion molecules in their blood, due to endothelial dysfunction; in these, ACE inhibition has been found to improve endothelial function, and reduce levels of VCAM-1 (Drexler *et al.*, 1995).

Having reached the interstitial space, the leucocytes must migrate towards the site of inflammation, travelling along a chemokine gradient. Monocyte chemoattractant protein-1 (MCP-1) is one such chemokine, and seems to play a central role in mediating Ang-II-induced vascular inflammation in rat models (Chen *et al.*, 1998). Looking at the situation *in vivo*, patients suffering a myocardial infarction have raised MCP-1 levels, which are reduced by ACE inhibition (Soejima *et al.*, 1999). RANTES (an α chemokine Regulated upon Activation, Normal T-Cell Expressed and Secreted) is another factor involved in leucocyte migration, and upregulated by Ang-II (Wolf *et al.*, 1997). As described earlier, atheromatous plaques are sites of chronic inflammation, containing focal areas of macrophages. Increased ACE and Ang-II expression has been

found in the macrophage-rich zones within an atheromatous plaque, providing some clinical evidence for the RAS being important in leucocyte attraction (Potter *et al.*, 1998; Diet *et al.*, 1996). Moreover, in experimentally-induced ACE deficient mice, a decrease in atherosclerosis has been observed (Hayek *et al.*, 2003).

3. Tissue repair

The final stage of the inflammatory response involves the clearing of debris and the start of tissue repair, by regeneration or fibrous scarring. Again, Ang-II has been implicated in these processes. Thus, Ang-II can directly upregulate the oxidative stress, and hence phagocytic activity of macrophages (Yanagitani *et al.*, 1999), clearing the way for growth and fibrosis to start. Indeed, differentiation of monocytes to activated macrophages is associated with activation of the RAS (Okamura *et al.*, 1999). Ang-II can also stimulate the cell proliferation and deposition of extracellular matrix proteins that is required for successful healing (Mezzano *et al.*, 2001). This has been demonstrated in vascular cell models (Johnson *et al.*, 1992), with VEGF and TGF-β being implicated as possible mediators for this (Otani *et al.*, 1998; Wolf *et al.*, 1993).

Thus overall, ACE has been shown to play a pro-inflammatory role in virtually every stage of acute inflammation. Turning to the systemic response to inflammation, an association between the RAS and other known pro-inflammatory cytokines has been established. For example, Ang-II has been shown to increase production of the early pro-inflammatory cytokine TNFα, (Klahr *et al.*, 1998). In rats, infusion of Ang-II upregulated renal expression of TNFα (Ruiz-Ortega *et al.*, 2002). More significantly, hypertensive patients have elevated TNFα levels (Bautista *et al.*, 2005), which fall following treatment with an ACE inhibitor (Suzuki *et al.*, 2003). Similarly, consistently

lower TNFα levels have been reported in chronic renal failure patients treated with ACE inhibitors (Stenvinkel *et al.*, 1999). As described in Section 1.1, another of the main systemic cytokines is IL6, and the relationship between it and the RAS has been the subject of much attention, since it may form the basis of the pro-inflammatory effects of ACE.

ACE and IL6

If ACE does possess the pro-inflammatory influence that the experimental evidence suggests (see Section 1.2.2), it would seem reasonable to postulate that it may also upregulate IL6. Hence, *in vitro* investigation of smooth muscle cell and macrophage cultures, demonstrated that Ang-II induces both IL6 gene transcription and protein expression (Schieffer *et al.*, 2000), an effect prevented by ACE inhibitors and AT1-receptor blockers (Kranzhofer *et al.*, 1999). This putative relationship is supported by the co-localisation of ACE, Ang-II, AT receptors and IL6 in coronary artery atheromatous plaques (Schieffer *et al.*, 2000). In addition, high-dose ACE inhibition in chronic heart failure patients has been associated with falls in systemic levels of IL6 (Gullestad *et al.*, 1999). Indeed, this apparent ACE inhibitor-mediated reduction in both ACE and IL6 may explain the ability of these drugs to prevent acute coronary syndromes, even at low doses (Montgomery, 2003). Looking at the post-surgical inflammatory response, a reduction in the stimulated rise of IL6 post-coronary artery bypass surgery has been observed with ACE inhibition (Brull *et al.*, 2002).

Thus far, the link between ACE and IL6 has been on the basis of association. However, much more convincing evidence comes from examining the possible mechanism underlying this interaction. IL6 transcription is controlled by a promoter that is the

target for cytokine-inducible transcription factors, with nuclear factor-κB (NF-κB) being the key one. So, the finding that Ang-II is able to activate NF-κB in human monocytes (Kranzhofer *et al.*, 1999) suggests that this pro-inflammatory transcription factor may drive the stimulated synthesis of IL6. Indeed, this mechanism has been confirmed in vascular smooth muscle cells (Han *et al.*, 1999), and the reciprocal relationship between IL6 and the RAS also demonstrated. In both *vitro* and *vivo* in mice, IL6 can induce upregulation of AT1-receptor gene expression, leading to increased Ang-II-mediated vasoconstriction and free oxygen radical generation (Wassmann *et al.*, 2004).

In summary, there is much evidence to suggest that ACE acts as a pro-inflammatory cytokine, and interacts positively with IL6 (summarised in Table 1.10). Moreover, in a similar way to IL6, inter-individual variation in both ACE levels and response to an inflammatory stimulus may be due to the existence of a common functional polymorphism, which will be investigated in the next section.

Inflammatory phase	Effect	Ref.
Vascular	- Ang II stimulates synthesis and secretion of VEGF in human cells	Pupilli *et al*, (1999)
Vascular	- VEGF can induce ACE production in human cells	Saijonmaa *et al*, (2001)
Leucocyte	- Expression of endothelial ICAM-1 and VCAM-1, and leucocyte integrins by Ang II in rat models	Tummala *et al*, (1998)
Leucocyte	- In chronic heart failure patients, ACE inhibition reduces levels of VCAM-1 and improves endothelial function	Drexler *et al*, (1995)
Leucocyte	- ACE inhibition reduces levels of chemokine MCP-1 in myocardial infarction	Soejima *et al*, (1999)
Leucocyte	- Finding of increased ACE and Ang II expression in macrophage-rich zones within human atheromatous plaques	Potter *et al*, (1998)
Tissue Repair	- Ang II upregulated oxidative stress and phagocytic activity of macrophages from human leukaemia cell line	Yanagitani *et al*, (1999)
Tissue Repair	- Stimulation of cell proliferation and extracellular matrix protein deposition by Ang II in rats	Johnson *et al*, (1992)

Table 1.10 Summary of pro-inflammatory effects of ACE

ACE has been shown to influence almost every phase of the acute inflammatory response.

1.4.3 ACE Insertion/Deletion Polymorphism

Despite having such widespread effects, the routine measurement of ACE levels has been of little clinical relevance. One of the exceptions to this is the inflammatory granulomatous disease sarcoidosis; plasma ACE levels are often elevated with this condition, so measurement forms part of the diagnosis and follow up (Lieberman, 1975). However, the practical application of this observation is hindered by the large inter-individual variation in plasma ACE levels that exists. This variation was found to be less prominent within families (Cambien *et al.*, 1998), suggesting a genetic basis of the variation.

It was on this background, that the investigation of the role of the ACE gene in determining circulating ACE levels was commenced. The ACE gene is found on chromosome 17q23, and consists of 26 exons (Hubert *et al.*, 1991), yielding an 83.3kDa protein. Two forms of ACE have been described. The more prevalent somatic ACE, is transcribed from exons 1-26, excluding exon 13, contains two active domain sites and is primarily expressed in endothelial cells. In addition, there is testicular ACE, which is transcribed from exons 13-26, only contains one of the active domain sites, and is expressed in the male reproductive tract (Hubert *et al.*, 1991). Investigation of inter-individual variation in plasma ACE levels led to the discovery of a polymorphism consisting of the presence or absence of a 287 base pair (bp) DNA fragment within intron 16 of the ACE gene (Rigat *et al.*, 1990), as illustrated in Figure 1.7. So, possession of this insertion polymorphism in neither, one or both alleles of the gene was termed D/D, I/D and I/I respectively. Moreover, this insertion/deletion (I/D) polymorphism was found to demonstrate mendelian inheritance, and be responsible for

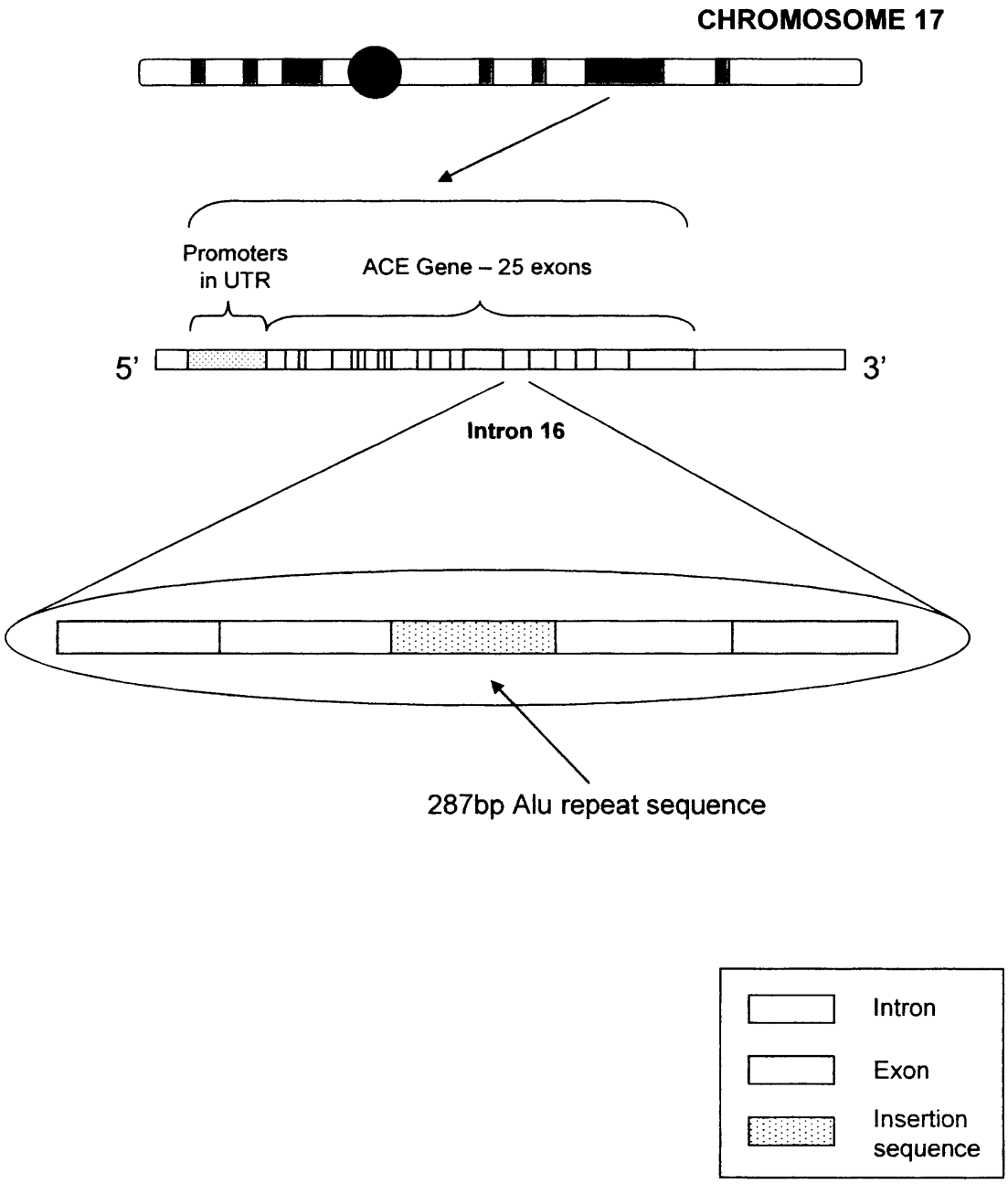


Figure 1.7 Schematic of the ACE gene with insertion polymorphism

UTR, untranslated region. The ACE gene lies on the short arm of chromosome 17. The I/D polymorphism is determined by the presence or absence of a 287bp Alu repeat sequence within intron 16.

47% of the variance in serum ACE levels, and was thus functional, with possession of the D-allele responsible for higher ACE levels. Closer analysis of the polymorphic insertion sequence showed it to correspond to an *alu* repetitive sequence (Rigat *et al.*, 1992). *Alu* sequences are insertional elements which account for about 10% of the human genome and possess a dimeric structure (Rowold *et al.*, 2000). Although genetically functionless, these repeat sequences can nevertheless affect the expression of neighbouring parts of the genome, by a number of mechanisms (Rowold *et al.*, 2000). One such mechanism involves the insertion of part of the *alu* sequence into mRNA by a process of "exonization", resulting in altered gene function (Lev-Maor *et al.*, 2003). It is thus entirely possible, despite lying within an intron, for the I/D polymorphism within the ACE gene to be functional.

1.4.4 Functional Implications of I/D Polymorphism

The developing importance of the ACE I/D polymorphism was underlined by subsequent work which demonstrated it to be functional in mononuclear cells (Costerousse *et al.*, 1993) and tissue (Danser *et al.*, 1995) as well as systemically. Thus D/D subjects have consistently higher levels of tissue and circulating ACE than I/D and I/I subjects. Further supporting evidence was provided by the finding that ACE gene transcription (as measured by mRNA transcript levels) has been associated with the I/D polymorphism in renal tissue, with I/I homozygotes having the lowest levels (Mizuiri *et al.*, 2001). In addition, a study of I/D heterozygotes, which is discussed in much greater detail in Section 3.7, demonstrated that the D-allele contributes a relatively greater degree of gene transcription compared to the I-allele (Suehiro *et al.*, 2004).

Due to the widespread distribution and influence of the RAS, and the influence of the polymorphism on ACE, the possible implications for both physiology and pathology have been the focus of much investigation (Woods *et al.*, 2000). This has often taken the form of association studies, which compare genotype distributions in control and test populations, with the aim of establishing an increased risk of disease with a particular genotype. A few of the main topic areas relevant to the surgical patient are discussed.

ACE and cardiovascular disease

The interest in the RAS and cardiovascular disease stems from both systemic and local function. Thus, the endocrine RAS is involved with blood pressure regulation, as described previously, and the local myocardial RAS has been implicated in cell growth (Higashimori *et al.*, 1993) and cardiac myocardium remodelling (Swynghedauw *et al.*, 1999). Consequently, pharmaceutical manipulation of the RAS has become successfully established for the treatment of a variety of cardiac diseases. However, there is a definite inter-individual variability in the responses to such treatments, which has been partly attributed to genetic polymorphism within the RAS, affecting circulating levels of ACE (Cicoira *et al.*, 2001). This in turn leads to the question of whether genetic polymorphism directly affects cardiovascular function.

The first report of an association between the ACE I/D polymorphism and disease was in the assessment of myocardial infarction (MI), with the D/D genotype being a risk factor for atherosclerosis, thrombosis and vasoconstriction (Cambien *et al.*, 1992). However, subsequent meta-analysis and larger studies (Keavney *et al.*, 2000) have not reproduced this finding, with the D/D genotype being only a very low independent risk

factor for MI. More recently, it has been suggested that the ACE polymorphism may provide prognostic information in post-MI patients, but only in combination with other established factors, such as left ventricular ejection fraction (Palmer *et al.,* 2003). Similarly, despite early reports of an association between the D/D genotype and cardiomyopathy with progression of heart failure (Raynolds *et al.,* 1993), this finding has not been consistently replicated (Tiret *et al.,* 2000).

The study of genotypic association with left ventricular hypertrophy (LVH) has proved to be no more successful. The knowledge that Ang-II causes cardiac hypertrophy (Higashimori *et al.,* 1993) underlay the search for gene association. The first report concluded that a positive association existed between D/D genotype and LVH, as diagnosed on electrocardiographic criteria, (Schunkert *et al.,* 1994). This is in line with the fact that the I/D polymorphism is associated with ACE gene expression in the left ventricle (Davis *et al.,* 2000), and animal studies which have demonstrated a regulatory role for the RAS in left ventricular hypertrophic responses (Lee *et al.,* 1993). However, once again there has been a wide variation in subsequent findings, with a meta-analysis failing to conclude an overall association between D/D genotype and LVH, except in untreated hypertensive subjects (Kuznetsova *et al.,* 2000).

Investigation of left ventricular growth (LV growth), rather than LVH, has been more conclusive. An association study of UK army recruits revealed a strong association between the D/D genotype and exercise-related LV growth, as measured by echocardiogram (Montgomery *et al.,* 1997). The homogeneity of this study population, especially when compared with the LVH patient cohort, may partly explain the positive results which have been consistently reproduced (Diet *et al.,* 2001). So, although few

clear associations between the ACE I/D genotype and cardiovascular disease have been made to date, the finding that the D-allele correlates with exercise related LV growth has encouraged the investigation of ACE and fitness.

ACE and physical fitness

It was actually the discovery of local tissue RAS acting as a positive growth factor within cardiac and skeletal muscle (Katz *et al.,* 1992), rather than the traditional view as a blood pressure modulator, which prompted an examination between human performance and the ACE I/D polymorphism. Thus, a preponderance of the ACE I-allele was observed amongst a cohort of elite mountaineers, and I-allele homozygotes (I/I) demonstrated greater improvements in endurance when performing a repetitive weightlifting exercise compared to D-allele homozygotes (D/D) (Montgomery *et al.,* 1998). This putative association between endurance performance and the ACE I-allele has been investigated in a multitude of other cohort groups, and their findings are summarised in Tables 1.9 and 1.10.

The findings from these gene association studies have not been uniform, with several failing to identify any association between ACE genotype and human performance in either athletes, or a general population (Taylor *et al.,* 1999; Rankinen *et al.,* 2000). The discrepancy with these studies may be explained by the choice of maximal oxygen uptake (VO_2max) as the endpoint marker of cardio-respiratory fitness, as individuals with the same VO_2max can differ in their endurance performance (Coyle *et al.,* 1988). Furthermore, more recent studies suggest that the ACE polymorphism does not mediate its' enhancement of endurance performance by improvements in VO_2max (Woods *et al.,* 2002).

Study Recruits	N°	End-point Measure	Conclusion	Ref.
Caucasian UK male army recruits	123	Max. duration of repetitive elbow flexion while holding 15kg barbell following 10 week general physical training program	I allele associated with improved endurance performance	Montgomery *et al,* (1998)
Australian Olympic Trial rowers – both sexes; Caucasian	64	Comparison of ACE polymorphism between elite rowers and matched controls	I allele frequency significantly increased in the rowers	Gayagay *et al,* (1998)
UK male army recruits; Caucasian	123	Changes in body composition as measured by bio-impedance, skin-fold thickness and MRI of mid-thigh following 10 week general physical training program	I/I genotype had a greater anabolic response than those with one or more D alleles	Montgomery *et al,* (1999)
UK Elite athletes selected as potential Olympians – both sexes & mixed disciplines	495	Comparison of ACE polymorphism between elite athletes and matched controls	I allele associated with improved endurance performance & increasing frequency with distance run	Myerson *et al,* (1999)
Elite and non-elite swimmers – both sexes; Caucasian	103	Comparison of ACE polymorphism between elite & non-elite swimmers and matched controls	D allele associated with elite short distance swimmers – effect diluted by including the non-elite swimmers	Woods *et al,* (2001)
Russian athletes labelled as outstanding or average – mixed sports & both sexes	217	Comparison of ACE polymorphism between athletes and matched controls – breakdown according to duration of event	D allele associated with outstanding short distance athletes, and I allele with outstanding middle distance athletes	Nazarov *et al,* (2001)
Elite long distance swimmers – ethnically diverse & both sexes	35	Comparison of ACE genotype frequency and swimming event longevity	I allele associated with longer distance swimmers, and D allele with shorter distance	Tsianos *et al,* (2004)

Table 1.11 Studies concluding a positive association between ACE genotype and physical performance

N°, number of patients.

Study Recruits	N°	End-point Measure	Conclusion	Ref.
Australian Elite aerobic athletes – both sexes & mixed sports; Caucasian	120	Comparison of ACE polymorphism between elite athletes and matched controls	No difference in ACE genotype frequencies between two groups	Taylor *et al,* (1999)
Male endurance athletes – mixed disciplines	192	Comparison of ACE polymorphism and VO_{2max} between elite athletes and matched controls	No association between ACE I/D polymorphism and elite endurance athlete status	Rankinen *et al,* (2000)
Sedentary subjects in good health – both sexes; Caucasian & Black	476	Change in VO_{2max} following personalised 20 week training program	No association between response to training & ACE genotype, except in Caucasian offspring – *D/D homozygotes* had best response	Rankinen *et al,* (2000)
US Army recruits – ethnically diverse & both sexes	147	Changes in VO_{2max} and performance in Army Physical Fitness Test (APFT) following 8 week basic physical training	No association between ACE genotype and VO_{2max} or APFT score, both before and after training	Sonna *et al,* (2001)
Finnish middle aged subjects – both sexes	455	Levels of self-reported moderate-intensity leisure time physical activity (MILTPA), BP and Hx of hypertension	No association between ACE genotype & MILTPAR, BP or hypertension	Fuentes *et al,* (2002)

Table 1.12 Studies concluding no association between ACE genotype and physical performance

N°, number of patients in study group. VO_2max, maximal oxygen uptake. BP, blood pressure.

Instead, a local effect of the ACE polymorphism within skeletal muscle has been identified, which produces significant improvements in contractile efficiency in I-allele homozygote army recruits in response to a specific training program (Williams *et al.*, 2000). A lack of homogeneity within the test cohort population may be another possible confounding factor within the studies with negative findings (Woods *et al.*, 2000).

As well as improved skeletal muscle contractile efficiency, enhanced metabolic efficiency, as demonstrated by greater anabolic responses and a relative sparing of fat stores in response to a training program, has been suggested as a possible underlying mechanism for the superior endurance response to training in I-allele individuals (Montgomery *et al.*, 1999). Interestingly and in contrast, an excess of the D-allele has been reported in elite sportsmen participating in short 'power' distance events (Nazarov *et al.*, 2001).

In summary, a body of evidence exists to suggest that the functional ACE I/D polymorphism has some influence on exercise performance, response to training, and metabolic efficiency. As will be discussed later in Section 1.5, this may be of relevance when considering the ability to tolerate surgery, given the knowledge that patient fitness correlates with outcome (Lee *et al.*, 2006).

ACE and inflammatory conditions

Given the pro-inflammatory role of ACE outlined in Section 1.3.2, the association between the I/D polymorphism and inflammatory conditions has been studied.

ARDS is characterised by increased pulmonary vascular permeability with subsequent inflammatory oedema and fibrosis. Given the established pro-inflammatory role of ACE, and its known localisation in the vasculature, it is perhaps unsurprising that ARDS patients have elevated levels of both ACE activity and Ang-II (Wenz *et al.*, 1997). Moreover, the ACE D/D genotype, with associated elevated plasma ACE protein levels, has been associated with the development of and mortality from ARDS (Marshall *et al.*, 2002).

In a similar way to ARDS, another condition where the magnitude of the patient inflammatory response relates to outcome is paediatric meningococcal disease (Westendorp *et al.*, 1997). The D/D genotype associated with a higher risk of mortality, longer intensive care stay and greater requirement for organ support – all reasonable end-point measures for poorer outcome (Harding *et al.*, 2002). Other inflammatory conditions associated with the ACE genotype include systemic lupus erythematosus (Pullman *et al.*, 1999), asthma (Urhan *et al.*, 2004), Parkinson's disease (Lin *et al.*, 2002) and juvenile rheumatoid arthritis (Alsaeid *et al.*, 2003).

In summary, the ACE I/D polymorphism has been shown to be functional, with the D-allele resulting in higher ACE gene transcription, protein levels and enzyme activity. Since the RAS has extensive systemic effects on blood pressure, and local effects on cell growth, much work has focused on the association between ACE genotype and a multitude of conditions, diseases and outcomes. Amongst these are patient susceptibility to cardiovascular disease, fitness, and recovery from inflammatory-driven critical illness – both of relevance to the peri-operative surgical situation. Despite this, the role of the ACE genotype in influencing individual surgical outcome has received

little attention compared to other cytokine gene polymorphisms. Nevertheless, those studies that have addressed this topic are described in the next section.

1.4.5 ACE and Surgical Outcome

Renal Transplantation Surgery

The RAS is a major determinant of blood pressure, and hypertension has been established as a major factor in transplanted end-organ injury (Marder *et al.*, 2003). Given this, and the established pro-inflammatory role of ACE, it is logical that polymorphisms within the RAS have been targets for investigation. An association between the D-allele and worse outcome has been suggested, in both kidney (Abdi *et al.*, 2001) and heart (Pethig *et al.*, 2000) transplant surgery. However, as with studies examining the -174 G/C IL6 polymorphism, this has not been a consistent finding (Nicod *et al.*, 2002), with a beneficial influence of D/D genotype also being found (Viklicky *et al.*, 2001).

General Surgery

There is only one other published study that has analysed the role of the ACE I/D polymorphism on surgical outcome, and this looked at 152 patients undergoing oesophagectomy (Lee *et al.*, 2005). This cohort of patients was chosen, because surgery remains the mainstay of treatment for potentially respectable tumours, yet the risk of post-operative morbidity and mortality remains high, affecting up to 35% of patients (Fan *et al.*, 1987). Moreover, the majority of these complications are pulmonary, and thought to be due to inflammatory responses within the lung tissue (Tsukada *et al.*, 2001). Consequently, potential genetic pre-disposition to developing such complications focused on ACE, due to its pro-inflammatory role, and the increased

susceptibility to ARDS (Marshall *et al.*, 2002) and asthma (Benessiano *et al.*, 1997) demonstrated by ACE D/D homozygotes. The main finding was that the D-allele was associated with a greater risk of developing pulmonary complications. However, despite finding that plasma ACE protein levels increased in a dose-dependent fashion with the presence of the D-allele, no association between elevated pre-operative ACE concentration and an increased risk of pulmonary complications was evident in these oesophagectomy patients.

So, there does seem to be evidence supporting a role for the ACE I/D polymorphism in influencing surgical outcome. However, only the relatively uncommon procedures of renal transplantation and oesophagectomy have been studied, and it is unclear how applicable these results are to the relatively more straightforward operations that form the bulk of general surgical practice. Patients undergoing minor procedures as a day-case or with short hospital stay, and a low risk of post-operative complications, will not be good subjects for such investigation, as they mount a smaller inflammatory response, which is less likely to affect outcome (Shenkin *et al.*, 1989). However, a suitable and large cohort undergoing major surgery, which will only increase in size with the introduction of the National Screening Plan (NHS Bowel Cancer Screening Program, 2006) is provided by colorectal cancer patients. A description of these patients, and the rationale for focusing investigation on them, forms the basis of the next section.

1.5 COLORECTAL CANCER SURGERY

1.5.1 Pathology, Indications and Complications

There are a large number of patients undergoing lower gastrointestinal oncological resection in the UK, because colorectal adenocarcinoma is a common disease, with around 35,000 new cases and 16,000 deaths annually. It is responsible for around 13% of adult malignant tumours, and is the second commonest cause of death from cancer in the UK (CRC Cancer Statistics, 2003). It is more common in North America and Western Europe than developing countries, an observation that is thought to be due to the greater prevalence of a high fat and low fibre diet, resulting in slower gut transit time, and increased mucosal exposure to bile salts (Meance *et al.*, 2003).

As with most malignancy, the underlying cause of colorectal cancer is unclear, but involves a combination of environmental and genetic factors. The former is evidenced by immigrant studies that show that migration from a low-incidence to a high-incidence region results in an increased incidence of colorectal cancer, to the same elevated levels as the indigenous population (Flood *et al.*, 2000). Other known environmental factors include pelvic irradiation (Jao *et al.*, 1987) and the presence of inflammatory bowel disease (Jess *et al.*, 2006).

Around 24% of cases of colorectal cancer have an identifiable family history, pointing to the importance of underlying genetic factors in this disease. The two main inherited conditions that have been implicated in the aetiology of colorectal cancer are Familial Adenomatous Polyposis and Hereditary Non-Polyposis Colon Cancer. However, these only account for a small proportion of family history cases, with the remainder likely to

be due to mutations in tumour suppressor genes (*eg* p53), oncogenes (*eg* RAS) and mismatch repair genes (*eg* hMSH2) (Jeter *et al.*, 2006).

Around 75% of cases are situated in the rectum and sigmoid colon, and assessment of suspected cases involves history, clinical examination and investigation with sigmoidoscopy, colonoscopy, double contrast barium enema and CT scan (Mitry *et al.*, 2002). Although only originally described for rectal tumours, a modified Duke's system has become one of the main accepted staging classifications in the UK (Dukes *et al.*, 1958):

Dukes' Stage	**Extent of tumour**	**5 year surviva!**
A	Confined to bowel wall	90%
B	Through bowel wall, but not lymph nodes involved	70%
C	Lymph nodes involved, but no other metastases	40%
D	Distant metastases	15%

The principle of elective surgery is wide resection of the tumour with regional lymphatics in curable cases, and the relief of potential or actual obstruction in the incurable cases (Mitry *et al.*, 2002). The presentation of colorectal cancer as an emergency is now less frequent, and is usually due to one of three main complications – obstruction, perforation or fistula formation; these are all associated with a poorer prognosis (McArdle *et al.*, 2004).

1.5.2 Outcome Determinants

The outcome from colorectal cancer surgery can be subdivided into the immediate post-operative period, and the longer term. Starting with the former, the factors that will determine outcome can be split into those intrinsic to the patient, and those that relate to the surgical care received.

Peri-operative outcome

It is well established for a wide variety of surgery that patient fitness influences outcome. More specifically, it is a patient's functional capacity, the difference between basal and maximal function, which correlates best with overall health status (Eagle *et al.,* 1996) and will govern outcome. Thus, those with a poor functional capacity will not be able to increase the delivery of oxygen to tissues to meet the extra demand provoked by the stress of surgery, and will consequently be at greater risk of post-operative complications (Gerson *et al.,* 1990). The ASA pre-operative scoring system is the simplest assessment of patient fitness and in combination with age has proved to be a good predictor of post-operative complications (Hall *et al.,* 1996).

Focusing on colectomy for colorectal cancer, predictors of peri-operative death have been found to be ascites, hypernatraemia and ASA III/IV scoring (Longo *et al.,* 2000). Looking at morbidity, as well as the cardiopulmonary and thromboembolic complications that associate with all operations, colorectal cancer surgery faces the specific potential problems of wound infection, anastomotic leakage and intra-abdominal abscess collection. This cohort of patients is at particular risk of wound infection due to the contamination inherent to the procedure, which is often compounded by the debilitated health status. However, the prophylactic use of

antibiotics has decreased the incidence of wound infection (Platell *et al.*, 2001). Obesity has been found to be a significant predictor of anastomotic leak (Biondo *et al.*, 2005). Another larger study concluded that use of an irrigation-suction drain, blood transfusion and a low anastomotic level (<5cm from anal verge) are independent risk factors for leakage (Yeh *et al.*, 2005). Similarly, pre-operative steroid use and longer operation time have been established as other risk factors for clinical anastomotic leakage (Konishi *et al.*, 2006). Turning to care-related factors, the specialisation of the surgeon (McArdle *et al.*, 2004) and the volume of work carried out within the hospital (Rabeneck *et al.*, 2004) both influence outcome in the short and long term; the latter being particularly true if the tumour is within the rectum.

Longer-term outcome

The question of outcome in terms of survival in the longer term following elective colorectal cancer surgery has also been much studied. Consequently a wide variety of factors influencing this have been suggested, such as male gender (McArdle *et al.*, 2003), low socio-economic status (Hole *et al.*, 2002), florid post-operative inflammatory response (McMillan *et al.*, 2003) and peri-operative anastomotic leakage (McArdle *et al.*, 2003). However, overall, it is the extent of the disease at the time of surgery, as quantified by Dukes' stage, which has been reported as the greatest determinant of long term survival (Angelopoulos *et al.*, 2004).

A role for genetic polymorphism?

However, despite reports of poorer outcome in Afro-Caribbean patients with colorectal cancer (Dominitz *et al.*, 1998; Alexander *et al.*, 2004), the potential influence of genetic polymorphisms on both short and long term outcome has not been studied. More

specifically, the inter-individual variation in post-operative inflammatory cytokine response, which has been associated with outcome, may be partly due to the IL6 -174 G/C SNP. Similarly, the functional I/D polymorphism may also affect the pro-inflammatory ACE response to surgery, and consequently outcome. In addition, given the emerging relationship between the ACE polymorphism and physical fitness, it may also modulate outcome by influence patient ability to tolerate the surgical insult.

In summary, there are several reasons why these colorectal cancer patients were chosen as the cohort to study:

- Common nature of the underlying adenocarcinoma pathology, meaning that a large number of patients exist as possible subjects for investigation, and may potentially benefit from this work.
- There is no evidence to suggest that cytokine gene polymorphisms are associated with the incidence of colorectal cancer, and there has not been any published work on whether they influence post-operative outcome.
- Surgical resection for adenocarcinoma remains the gold-standard treatment, and is likely to remain so.
- As a relatively major procedure, there is a relatively high risk of post-operative complications and variation in post-operative length of stay following colorectal resection.
- Restricting the cohort to those undergoing elective oncological resection, to the exclusion of inflammatory bowel disease and diverticulitis patients, limits the variation in cytokine baseline levels and response to surgery, and aims to provide as homogeneous a study population as possible.

CHAPTER 2

Aims and Experimental Design

CHAPTER 2

2.1 BACKGROUND

Improving surgical outcome is of increasing importance, and a multitude of strategies are employed to achieve this, including the introduction of novel surgical equipment, materials and techniques, such as the minimally invasive laparoscopic approach (Sauerland *et al.*, 2004). The other main area of focus has been the optimal identification of the high-risk surgical patient, using a variety of pre-operative tests, who will benefit most from rationed intensive care resources (Lee *et al.*, 2006). Despite this progress, the expected improvements in surgical outcome have not really materialised (Casson *et al.*, 2005).

Rather than concentrating solely on the ability to *tolerate* surgery, there has been increasing interest in the patient's inflammatory *reaction* to surgery, especially as this has emerged as an outcome predictor (Roumen *et al.*, 1993). This response to surgery comprises an initial pro-inflammatory acute phase cytokine response, followed by a period of immunosuppression, when anti-inflammatory cytokines predominate. Considerable inter-individual variation in the nature and scale of this inflammatory response exists and it has been suggested that this may be due to genotypic differences in the functional cytokine polymorphisms (Damas *et al.*, 1997). Of even greater significance is the possibility that these polymorphisms may also influence the development of post-operative complications, as evidenced by the observation of racial differences in surgical outcome (Bradley *et al.*, 2002).

More specifically, IL6 has emerged as a key cytokine, mediating the early pro-inflammatory phase and also having a role in triggering the subsequent

immunosuppression (Castell *et al.*, 1989). Moreover a common G/C functional polymorphism has been identified at position -174 within the IL6 gene promoter region, and its association with a wide variety of inflammation-based conditions has been investigated (Fishman *et al.*, 1998). Its influence on the reaction to surgery has only begun to receive attention, with the current focus being on procedures that provoke a particularly florid inflammatory response, such as organ transplantation (Muller-Steinhardt *et al.*, 20002), CABG (Burzotta *et al.*, 2001) and aneurysm surgery (Bown *et al.*, 2004). However, these are relatively uncommon operations, and the influence of the IL6 -174 G/C polymorphism on the inflammatory reaction to a general surgical procedure, such as laparotomy for colorectal cancer, has not yet been assessed.

As well as its well-documented role as a short and long term modulator of circulation and blood pressure, much evidence points to ACE also being a pro-inflammatory cytokine (see Section 1.2.2). Consequently, the association of the functional ACE I/D polymorphism with a range of conditions has also been investigated. In spite of this, its impact on the post-surgical inflammatory response has only been investigated in a limited number of cases. The D-allele has been associated with a poorer outcome following solid organ transplantation (Abdi *et al.*, 2001), although this has not been a consistent finding (Viklicky *et al.*, 2001; Slowinski *et al.*, 2004). Similarly, in the only other study to analyse the effect of this ACE polymorphism on surgical outcome, the D-allele has been linked to a greater risk of developing pulmonary complications following oesophagectomy (Lee *et al.*, 2005). However, this study was only associative, and only measured pre-operative plasma ACE levels, without making any analysis of the influence of surgery on ACE expression.

The most common method of investigating the link between a certain candidate functional gene polymorphism and surgical outcome is an association study, comparing prevalence between two populations. However, to further assess whether the gene in question is actually responsible for any observed effect, rather than being in linkage disequilibrium with another gene, one can examine its activity. The basis of such an evaluation is the central genetic dogma, as illustrated and discussed previously in Figure 1.5 and Section 1.2.1. Thus, the measurement of levels of transcription along with quantities of protein end product will give a reflection of gene activity. By quantifying the gene response to an environmental challenge, and combining this with polymorphism association data, a clearer picture of whether the candidate gene is of influence might emerge.

Elective surgery for colorectal cancer is a common procedure that is not restricted to specialist centres, and is likely to remain the gold-standard treatment for the foreseeable future. It is known to elicit an inflammatory response, and the rise in post-operative IL6 has been associated with outcome (Mokart *et al.*, 2002). Moreover, the importance of a homogeneous study population, all undergoing oncological rather than benign surgery is due to the different inflammatory responses demonstrated by these two groups (Syk *et al.*, 1999). Different IL6 levels at baseline and following colectomy have been reported from a study of Crohn's and carcinoma patients (Riche *et al.*, 1995). Despite this work on IL6, the effect of colorectal cancer surgery on ACE expression has not been documented, and furthermore, the influence of both IL6 and ACE genetic polymorphism on the inflammatory reaction has not been investigated.

2.2 HYPOTHESIS AND AIMS

The central hypothesis of this study was that the functional ACE and IL6 polymorphisms modulate the inflammatory response to colorectal surgery, and consequently associate with outcome. The aim was to delineate the IL6 and ACE response to elective colorectal surgery, in terms of gene activity and protein levels, and investigate the influence of the functional I/D ACE and -174 G/C polymorphisms on this reaction and post-operative outcome. More specifically, this thesis aimed to:

1. Investigate potential demographic, pathological and operative factors influencing outcome from elective colorectal cancer surgery.
2. Determine the baseline IL6 gene and protein expression in colorectal cancer patients, the response to surgery and the influence of the -174 G/C genotype on both.
3. Determine the baseline ACE gene and protein expression in colorectal cancer patients, the response to surgery and the influence of the I/D genotype on both. Furthermore, investigate the individual allelic response to surgery.
4. Evaluate the potential influence of ACE and IL6 genotypes on post-operative outcome, and evidence of polymorphic functionality.

To address each individual aim, the following experimental series was undertaken.

2.3 EXPERIMENTAL DESIGN

Approval was sought from the Joint University College London/University College Hospitals London Committees on the Ethics of Human Research prior to the recruitment of patients due to undergo elective laparotomy for colorectal cancer. Potential subjects were approached pre-operatively and written informed consent obtained.

To address the first aim of the thesis, a medical history was taken, including medication prescribed, and prospective evaluation of the post-operative period of recuperation was made, noting factors such as the duration of the operative procedure, the occurrence of any post-operative complications, and the overall length of stay in hospital.

To deal with the second aim set out for this thesis required the evaluation of patient IL6 genotype, using DNA, and an assessment of gene expression, in terms of RNA transcription and plasma protein concentration. It was possible to perform all the analyses required, from samples of peripheral blood. Therefore, a 10ml sample of blood was taken pre-operatively, and then repeat venesection performed 4 and 24 hours following surgery. Patients were then asked to return at 10 and 20 weeks following discharge, with repeat blood samples taken. From these samples, plasma and peripheral blood mononuclear cells (PBMNs) were separated. From the latter, DNA was extracted, with IL6 genotype established using polymerase chain reaction (PCR) and *NLA III* restriction enzyme digestion. Following the isolation of RNA from PBMNs, an RT reaction was performed, producing cDNA. Subsequently, a semi-quantitative RT-PCR method of assessing gene transcription was performed, using specifically designed primers, with the house-keeping gene GAPDH-3 used for normalisation. RT-PCR

products were separated by agarose gel electrophoresis and scanning optical densitometry (OD) used to assess band-intensity. Finally, plasma levels of IL6 protein were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). Analysis of gene expression was carried out at all time-points.

Addressing the third aim, involved identical samples and experimental techniques, except specific primers were used to establish ACE genotype from DNA. In addition, specific primers were also used to assess overall ACE transcription from RNA. The individual contribution from the I- and D-alleles was assessed by exploiting the linkage between this polymorphism and the exonic G/A SNP at position 2215. An *HAE II* restriction reaction was used to distinguish between alleles. Again, a sandwich ELISA with specific capture and detection antibodies for ACE was used to assess plasma protein levels, and assays for gene expression at all time-points were performed.

The final aim, investigating the possibility of ACE and IL6 genetic polymorphisms exerting some influence over outcome, involved combining the genotype and gene expression results with the prospectively gathered details of post-operative recovery that were obtained from the patient notes.

A summary of the experimental hypothesis is included in Figure 2.1.

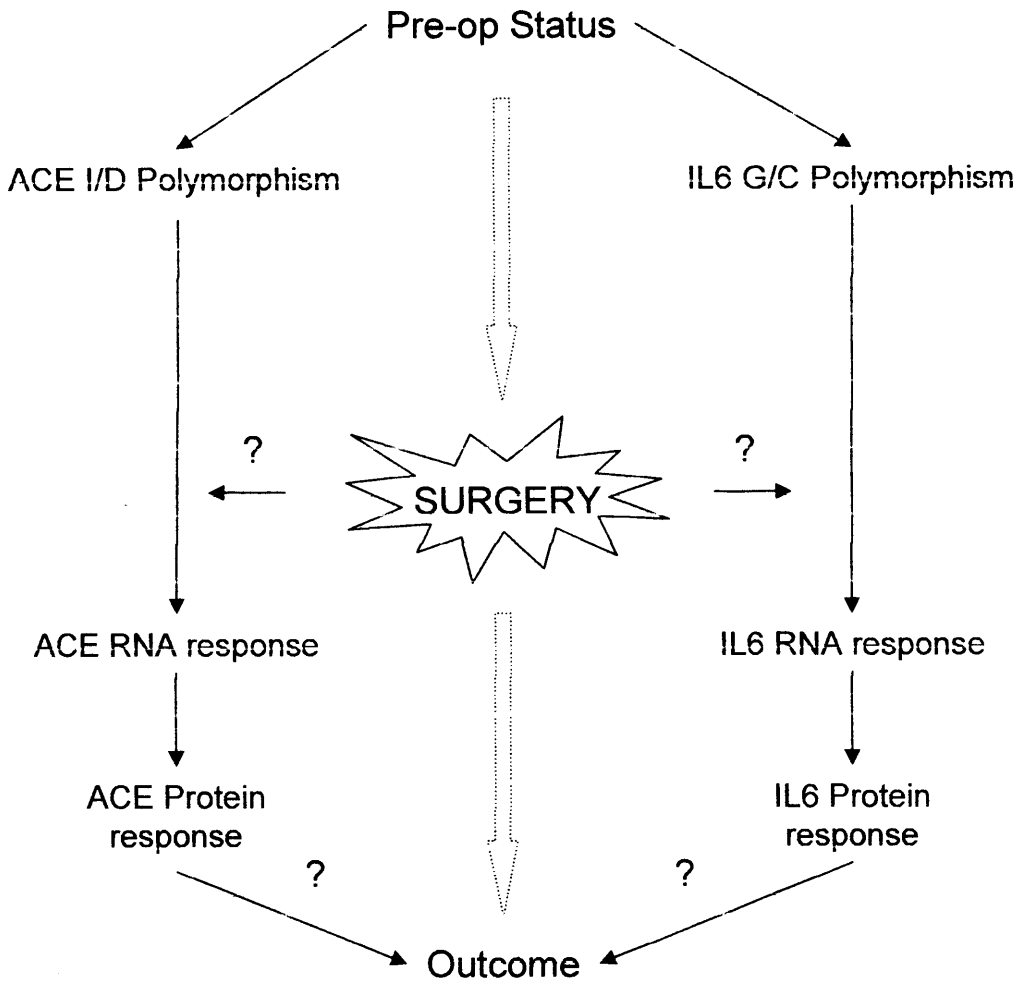


Figure 2.1 Hypothesis

The influence of the ACE I/D and IL6 -174 G/C polymorphisms on RNA and protein responses to colorectal cancer surgery are unknown. In addition, the functional effect of these responses on the clinical outcome from surgery is also unknown. Thus, the aim of this thesis was to investigate the influence of the functional ACE and IL6 polymorphisms on the inflammatory response to colorectal surgery, and consequent clinical outcome.

CHAPTER 3

Materials and Methods

CHAPTER 3

3.1 PROJECT SET-UP

3.1.1 Ethical Approval

The first step was to gain approval for the study from the Joint University College London/University College Hospitals London Committees on the Ethics of Human Research. This study began in 2003, before the introduction of the standardised Centralised Office for Research Ethics Committees (COREC) forms, which are now used nationwide for all applications to conduct research involving humans. The relevant local application form was completed, along with the submission of a Patient Information Sheet and Patient Consent Form. This application was considered by the Joint UCL/UCLH Committees on the Ethics of Human Research: Committee A, and following some minor amendments to the Patient Information Sheet, ethical approval was granted. A summary of the proposed study protocol, along with the patient inclusion and exclusion factors are shown overleaf in Figure 3.1. "Laparotomy" was a rather all-embracing term used to include the following procedures, tabled below and illustrated schematically in Figure 3.2.

Surgical procedures undergone by study patients	
Right Hemicolectomy	Subtotal Colectomy
Left Hemicolectomy	Anterior Resection of the Rectum
Transverse Colectomy	Abdomino-Perineal Resection of the Rectum
Sigmoid Colectomy	

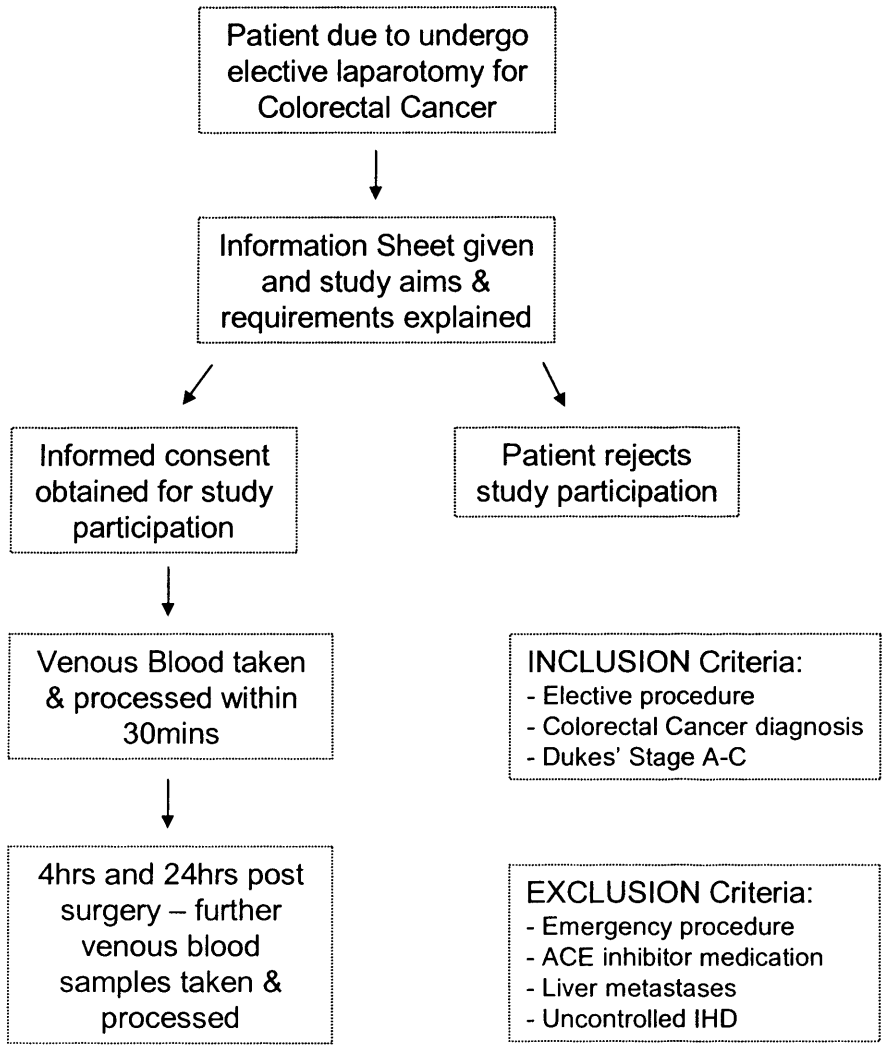


Figure 3.1 Protocol for study

Proposed study protocol, including patient inclusion and exclusion criteria. IHD, ischaemic heart disease.

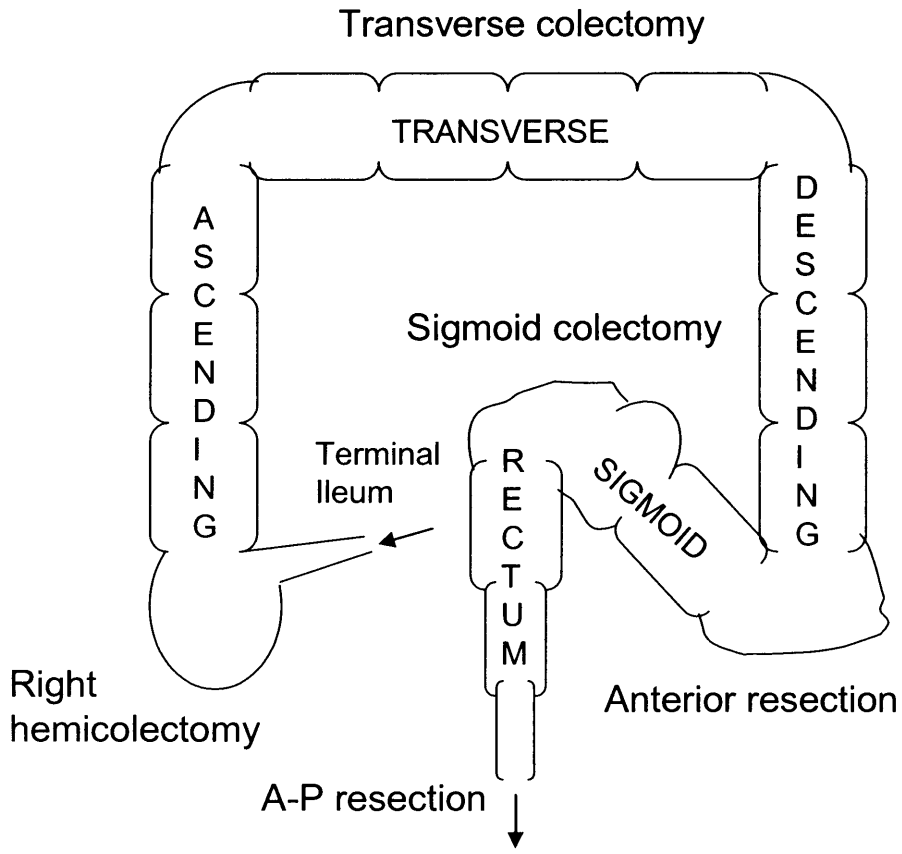


Figure 3.2 Schematic illustration of the colon

The colon is divided into anatomical segments which form the basis of the different types of surgical resection undergone by colorectal cancer patients. A-P resection, abdomino-perineal resection.

These surgical procedures do vary in their extent in terms of dissection, resection and operation time. Ideally, one would carry out the study by looking at patients undergoing one particular standard operation, carried out by the same surgical team, with the same anaesthetist and in the same institutional setting. However, a review of the surgical caseload within UCLH in the year 2002 (preceding the start of this study), summarised below, demonstrated that no single colorectal operation was performed often enough to be the sole focus of a study.

Audit of colorectal surgical procedures performed in 2003 at UCLH			
Procedure	**Number**	**Procedure**	**Number**
Right Hemicolectomy	20	Subtotal Colectomy	2
Left Hemicolectomy	4	Anterior Resection	17
Transverse Colectomy	0	Abdomino-Perineal Resection	10
Sigmoid Colectomy	3		

Combining this with the constraints of time meant that the decision was made to examine all patients with colorectal cancer undergoing surgery. There was no evidence to suggest a predisposition of any particular ACE genotype to a specific location of colorectal cancer, and so no reason to suppose that genotype may influence the type of procedure undergone. As the study was aimed at examining the possible genetic influences on the recovery from surgery, it seemed more important for the study population to have the same fundamental pathological diagnosis, at the expense of a slight variety in surgical procedure.

3.1.2 Research and Development Approval

Before patients could start being recruited for the study, approval had to be sought from the local Research and Development Committee at University College London Hospitals NHS Trust. This process also entailed ensuring that the study met the demands of the Data Protection Act (1998), with respect to the plans made for storing patient information; all data was kept on a password-protected computer database, within a double locked office. Furthermore a coding system was employed, making it impossible to link experimental results with patient identifiers.

3.1.3 Patient Recruitment

Potential eligible study subjects were identified with the help of the colorectal cancer nurse specialist, and approached whilst an in-patient on the ward, prior to surgery. If the patient had already commenced taking a laxative to prepare the bowel prior to surgery, then they were excluded from the study, as it was felt that this may influence the findings from any pre-operative blood sample. Following a clear and thorough explanation to both the patient, and any relatives present, regarding the nature, aims and demands of the study, an information sheet was offered and a short period of time left for deliberation. If the patient was keen to participate in the study, then any final queries were addressed, and they were asked to complete and sign a consent form.

3.2 SAMPLE COLLECTION AND PROCESSING

3.2.1 Sample Collection

Informed consent was obtained from the patient, and a standard aseptic technique was then employed to perform a peripheral venepuncture, with 10mls of blood being collected in Vacutainer® EDTA Blood tubes (Becton Dickinson, US). These samples were then immediately transported to the laboratory for processing within 30 minutes of collection.

3.2.2 Whole Blood Processing

The aim of processing is to separate out the plasma, leucocytes and erythrocytes, allowing the isolation and separate collection of these blood components. This is most commonly achieved by mixing the blood with a compound which only aggregates erythrocytes, thus increasing their rate of sedimentation. So, after a period of being centrifuged, the erythrocytes will settle at the bottom of the tube, whereas the relatively unaffected leucocytes will collect further up. Lymphoprep™ (AXIS-SHIELD, Norway) kept at room temperature and out of direct sunlight, was used to process the blood samples. It consists of a solution containing 9.1% Sodium Diatrizoate and 5.7% Polysaccharide, and, importantly, is both sterile and endotoxin tested.

Throughout the following protocol, and for all the rest of this chapter, all episodes of vortexing were performed with a bench-top Vortex-Genie™ (Scientific Industries, US). Centrifuging tubes was performed using an ALCPK120 centrifuge (Jencons Ltd, UK).

3.2.3 Whole Blood Processing Protocol

Step 1- Separation of blood components by differential centrifugation

A 3ml sterile Pasteur pipette (Becton-Dickinson, France) was used to carefully place 3ml of Lymphoprep™ into two sterile 15ml (Corning, USA) centrifuge tubes. Using another Pasteur pipette, 5mls of the anti-coagulated blood was carefully layered on top of the Lymphoprep™, ensuring a minimal amount of mixing. The two tubes were then spun at 400 x g for 30 minutes at room temperature. This produced a separation of the blood into distinct component layers, as illustrated in Figure 3.3.

Step 2 – Extraction of Plasma

The upper layer of plasma, which collected on top of the Lymphoprep™, was aspirated and then placed into another 15ml centrifuge tube. This was centrifuged again at 800 x g for a further 5 minutes, to pellet out any cellular debris. The supernatant was taken and aliquoted into sterile 1.5ml Eppenforf tubes (Starsted, UK). These were immediately placed into the -20°C freezer for storage.

Step 3 – Extraction of Peripheral Blood Mononuclear cells

At the blood sample/Lymphoprep™ interface a distinct band of peripheral blood mononuclear cells (PBMNs) collected, called the "Buffy Coat" layer. This was carefully aspirated from the interface using a Pasteur pipette and placed into another 15ml centrifuge tube containing 3mls of Phosphate Buffered Saline (PBS) (Sigma®, UK), to wash. The tube was filled up to 10mls with more PBS, and then centrifuged at 250 x g for 10 minutes to re-pellet the mononuclear cells. The supernatant was taken off and discarded, and the pellet of cells re-suspended in another 10mls of PBS.

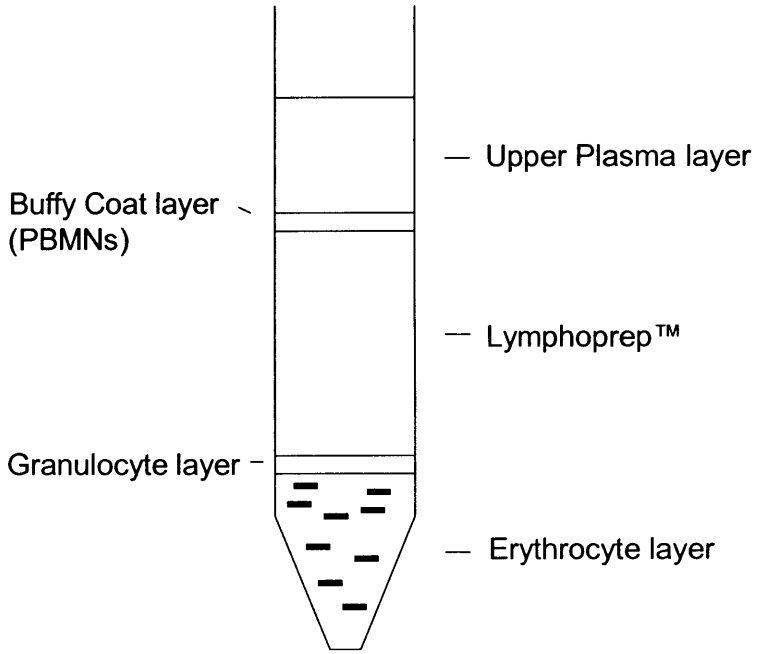


Figure 3.3 Representation of Lymphoprep™ separation

After centrifugation, the upper plasma layer stays above the Lymphoprep™. PBMNs collect at the plasma/Lymphoprep™ interface. Granulocytes collect as a fine layer on top of the erythrocytes.

After another 10 minutes of spinning at 250 x g, the supernatant was again carefully removed, and 200µl of PBS added to the washed cell pellet. This pellet was re-suspended by gentle agitation, and then 3mls of Tri-Reagent™ (Sigma®, UK) added to the tube. Following 10 seconds of vortexing, the mononuclear cells in Tri-Reagent™ were aliquoted into 3 separate sterile 1.5ml Eppendorf tubes and placed into the -20°C freezer for storage. The purpose of the Tri-Reagent™ will be discussed later.

Step 4 - Extraction of Granulocytes

Granulocytes gather at the erythrocyte pellet/Lymphoprep™ interface. Again, a 3ml Pasteur pipette was used to carefully remove this fine layer. The fine nature of this tier made it impossible to remove without inadvertently also taking some of the erythrocyte layer. To wash the granulocytes, they were placed in a 15ml centrifuge tube containing 10mls of a lysis buffer (1.55M NH_4Cl, 100mM $KHCO_3$, 1mM EDTA), aimed at minimising contamination with erythrocytes. The lysis buffer was made up using de-ionized water that had been filtered and UV treated by a Simplicity 185™ (Millipore, UK) machine to produce water with a resistivity of 18.2 MO at 25°C. This same de-ionized water was used for all the experimental protocols employed during this study, and shall henceforth be referred to as "ultrapure water".

This was left at room temperature for at least 10 minutes to ensure complete lysis. A further 10 minutes of centrifugation at 250 x g produced a pellet of granulocytes, with minimal erythrocyte contamination. The supernatant was taken and discarded before the granulocytes were re-suspended in 10mls of PBS for a final wash. After a final 10 minute spin at 250 x g, the granulocyte pellet was re-suspended in 200µl of PBS and placed into the -20°C freezer for storage.

3.3 EXTRACTION OF NUCLEIC ACIDS FROM PERIPHERAL BLOOD MONONULCEAR CELLS

3.3.1 Theory

The PBMNs isolated from blood samples were placed in Tri-Reagent™, because this mixture of guanidine thiocyanate and phenol enables the simultaneous extraction of cellular RNA, DNA and protein. This extraction protocol is based on the single step method for total RNA isolation (Chomczynski *et al.*, 1987). The Tri-Reagent™ effectively lyses the PBMNs, and the guanidine thiocyanate and phenol dissolves both protein and nucleic acids. This mixture is separated into three phases by the addition of chloroform and subsequent centrifuging:

- Upper aqueous phase, containing RNA
- Interphase, containing DNA
- Lower organic phase, containing protein

These three phases can be separated, allowing the isolation of the different components.

3.3.2 Protocol

The protocol employed was based on the manufacturer's guidelines (Sigma®, UK). A bench-top Genofuge 16 centrifuge (Techne Ltd, UK) was employed where appropriate.

Step 1 – Separation into 3 phases by differential centrifugation

Samples were removed from the freezer and allowed to thaw. They were then left at room temperature for at least 5 minutes before the addition of 200µl of chloroform

(BDH Laboratory Supplies, UK) to each 1.5ml Eppendorf tube, containing around 1ml of Tri-Reagent ™. This mixture was vortexed for 15 seconds before being left to stand for a further 10 minutes at room temperature. The tubes were then spun at 12,000 x g for 15 minutes – with the mixture separating out into the 3 phases described above and illustrated in Figure 3.4.

Step 2 – RNA Precipitation

The upper colourless aqueous phase was taken and transferred to a fresh Eppendorf tube. To precipitate the RNA out of solution, 0.5mls of isopropranolol (BDH Laboratory Supplies, UK) was added, and the tube left for 10 minutes to stand at room temperature before being centrifuged at 12,000 x g for 15 minutes. An RNA pellet formed at the bottom and sides of the tube, and with this in mind, supernatant was carefully removed, and the pellet washed in 1ml of 75% ethanol (BDH Laboratory Supplies, UK). The RNA, in ethanol, was vortexed and then centrifuged at 7,500 x g for 5 minutes to reform the pellet. Finally, the ethanol supernatant was removed, and the pellet allowed to dry out (but not completely), before the RNA was re-dissolved in 30µl of ultrapure water and placed into the -20°C freezer for storage.

Step 3 – DNA Precipitation

0.3ml of 100% ethanol was added to the remaining interphase and red organic phase in the original eppendorf tube. The tube contents were gently mixed by inversion and left to stand at room temperature for 3 minutes. Following 5 minutes of centrifuging at 2000 x g, a DNA pellet was formed at the bottom of the tube. The organic red supernatant was removed, and stored at -20°C for possible protein isolation at a later date.

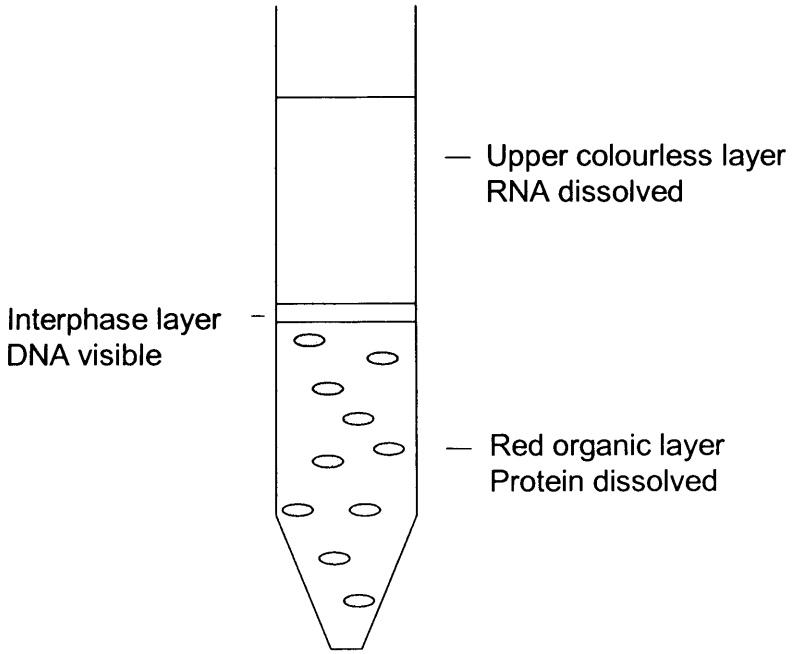


Figure 3.4 Representation of Tri-Reagent™ separation of nucleic acids

After centrifugation, there is separation into two distinct phases. The upper colourless phase contains dissolved RNA, and the lower red organic phase contains dissolved cellular proteins. DNA collects at the interface between the two.

It was important to remove as much of this phenol phase as possible, as it would interfere with later processing. 1ml of 0.1M sodium citrate, 10% ethanol wash solution was added to the remaining DNA and left for 35 minutes to stand at room temperature. After 5 minutes of centrifuging at 4000 x g to re-pellet the DNA, this wash step was repeated once more. 1ml of 75% ethanol was added to the washed DNA pellet, and the tube left to stand on the bench for 20 minutes. After a final 5 minute centrifuge at 4000 x g, the supernatant was removed, and the DNA pellet left to completely dry out. Once dry, the DNA was re-dissolved in 100µl of ultrapure water and placed into the -20°C freezer for storage.

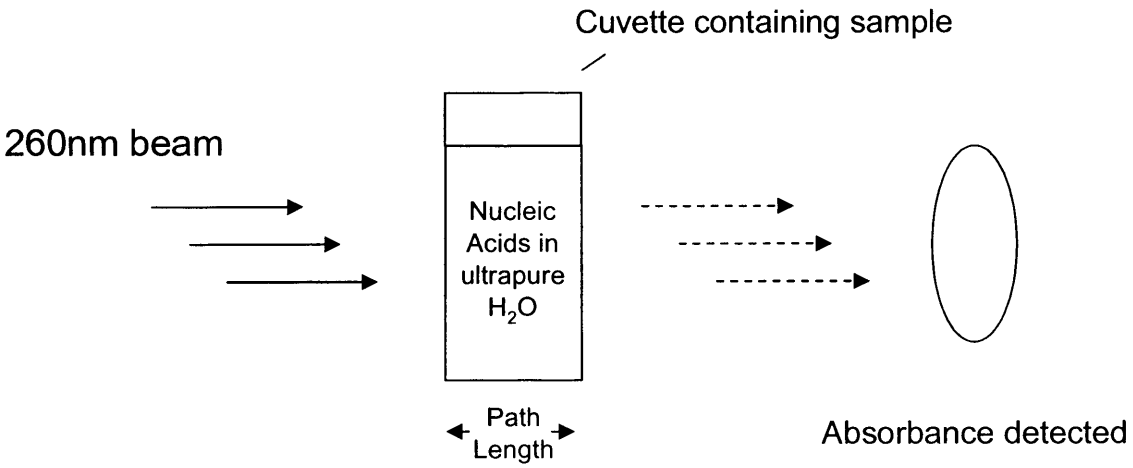
3.3.3 Quantifying Yields - Spectrophotometry

The concentration of dissolved DNA and RNA was established using spectrophotometry. This is based on the knowledge that nucleotides in solution (dATP, dTTP, dGTP and dCTP) will absorb light in the ultraviolet region of the spectrum – an effect which is maximal at a wavelength of 260nm. Using the Beer-Lambert Law, it is possible to calculate the concentration of the nucleotides in solution:

$$A_{?} = e_{?}bC$$

$A_{?}$ = Absorbance (in this case at 260nm)
$e_{?}$ = Absorptivity co-efficient of the material at specific wavelength (260nm)
b = Path length through the sample
C = Concentration of the sample

Thus nucleic acid concentration from samples was determined using a WPA Biotech (UK) spectrophotometer, as illustrated in Figure 3.5.



Protocol employed:

1. 147µl of ultrapure H_20 into cuvette
2. Spectrophotometer calibrated to zero
3. 3µl of nucleic acid into cuvette – making a total volume of 150µl
4. Absorbance at 260nm wavelength measured
5. Concentration of DNA/RNA calculated

Figure 3.5 Representation of spectrophotometry

Concentration of nucleic acids is established using a spectrophotometer to measure absorbance of light at 260nm. The protocol used involved a 1:50 dilution of the sample in ultrapure water.

3.4 ESTABLISHING PATIENT ANGIOTENSIN CONVERTING ENZYME GENOTYPE

3.4.1 Background

The functional Insertion/Deletion (I/D) polymorphism in the ACE gene was first described by a team looking for a genetic explanation for the natural variation in circulating ACE plasma levels found in normal subjects (Rigat *et al.*, 1990). An endothelial ACE cDNA probe was used, along with a series of restriction enzymes, to identify the I/D polymorphism at the ACE gene locus. This polymorphism was subsequently found to demonstrate Mendelian inheritance and to account for 47% of the total variance of serum ACE. Subsequently, a more convenient method of using the polymerase chain reaction (PCR) to detect the I/D polymorphism was described (Rigat *et al.*, 1992).

3.4.2 Polymerase Chain Reaction

PCR is an *in vitro* method of amplifying a nucleic acid sequence, without the need to employ bacteria to clone it. Moreover, this technique is able to quickly produce millions of copies of the target sequence, as its cyclical nature results in an exponential amplification. Thus, despite only having a limited amount of DNA available for analysis, one is able to amplify the relevant DNA sequence to such an extent that it is possible to macroscopically visualise and distinguish between the two different alleles of the ACE I/D polymorphism. PCR relies on the specific nature of purine-pyrimidine base pairing to produce faithful copies of the target sequence. PCR also requires knowledge of the nucleotide sequences flanking the region of interest that is to be amplified, and oligonucleotides complementary to these are used as primers. These

primers act as initiators for the specific series of heat-stable DNA Polymerase-catalysed reactions that comprise a PCR.

Each cycle of the PCR consists of 3 separate steps:

The 3 separate steps of the Polymerase Chain Reaction		
Step 1	Denaturation	Heat is used to denature the double-stranded DNA, separating it into its two component strands – exposing the normally well-protected nucleotide bases
Step 2	Annealing	The reaction is subsequently cooled to an optimal temperature for the binding of the oligonucleotide primers to their complementary DNA sequences
Step 3	Elongation	Reaction temperature is then raised back to a level for optimal DNA polymerase action – synthesizing a new DNA strand in a 5' to 3' direction using the primers as initiating "springboards" and the existing strand as a template

At the end of a cycle, a double stranded sequence of DNA is produced, consisting of the original template, and a newly synthesised DNA strand. The high temperatures required to denature the DNA will also result in the denaturation and inactivation of normal human enzymes, including DNA polymerase. Thus for the next repeat cycle of PCR, it would seem necessary to re-introduce more DNA polymerase into the reaction; an unwieldy, cumbersome and slow process. However, this is obviated by the use of a thermostable form of DNA polymerase from a bacterial species, such as *Thermophillus Aquaticus*, that lives at high temperatures and is consequently not inactivated at DNA-denaturing temperatures.

The first 3 cycles of a PCR reaction are illustrated in Figures 3.6 to 3.8. As can be seen, after each cycle, the number of copies of the original target DNA sequence will double – an exponential growth. So, after the 30 to 40 cycle repeats that comprise most standard PCR reactions, there will be millions of copies of the sequence of interest – enough to be potentially visualised macroscopically. Clearly, the amount of starting material will determine the number of transcripts at the end of the amplification process. This is only significant if one is trying to make an assessment on the number of transcripts present – which is the case when performing a RT-PCR reaction to measure gene transcription (discussed at length in Section 3.6). In the case of establishing genotype, it is only important to know if the PCR product is present or not – regardless of its intensity. Having discussed PCR in general terms, the specific use of PCR to determine ACE I/D genotype will be described.

3.4.3 Polymerase Chain Reaction – Primers

The choice of PCR primers will determine the specific DNA sequence that is to be amplified, and consequently the length of the resulting PCR product. Knowledge of the ACE gene sequence has allowed the design of primers such that the target sequence is within intron 16 of the ACE gene, and spans the polymorphic section that potentially includes the 287 bp "insertion". Thus, the presence or absence of this "insertion" sequence is detectable, based on the size of the product that is amplified by PCR – as illustrated in Figure 3.9. Clearly, if the "insertion" sequence is present, then the DNA sequence amplified by the primers will be longer, and this will be evident when the PCR products are resolved by agarose gel electrophoresis; a process of separation by charge and size, which is discussed in more detail in Section 3.4.7.

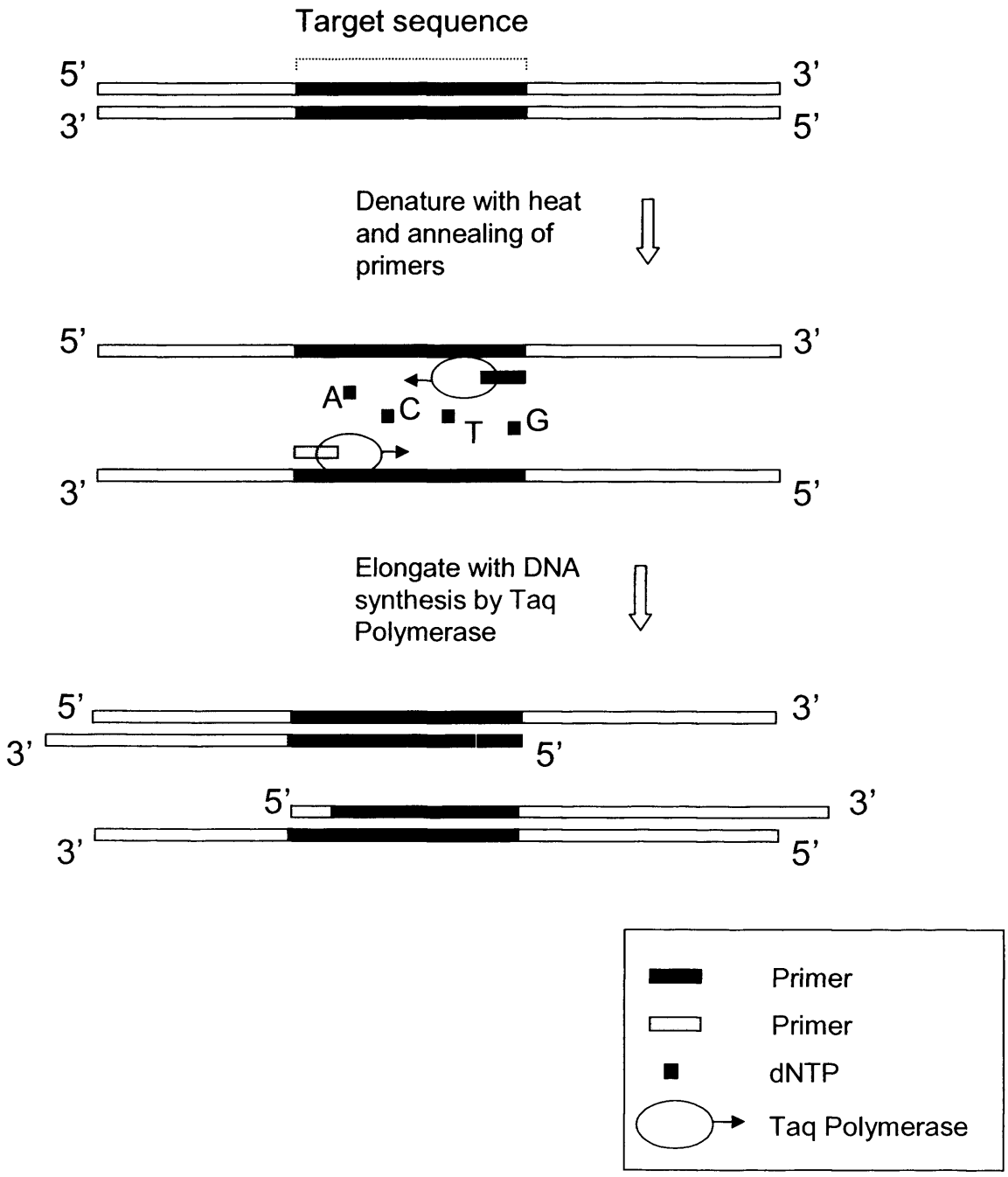


Figure 3.6 Cycle 1 of Polymerase Chain Reaction

Heat initially denatures and separates the two nucleic acid strands, allowing the primers to anneal, and provide a start-point for the Taq DNA polymerase to assimilate dNTPs into a complementary nucleic acid strand.

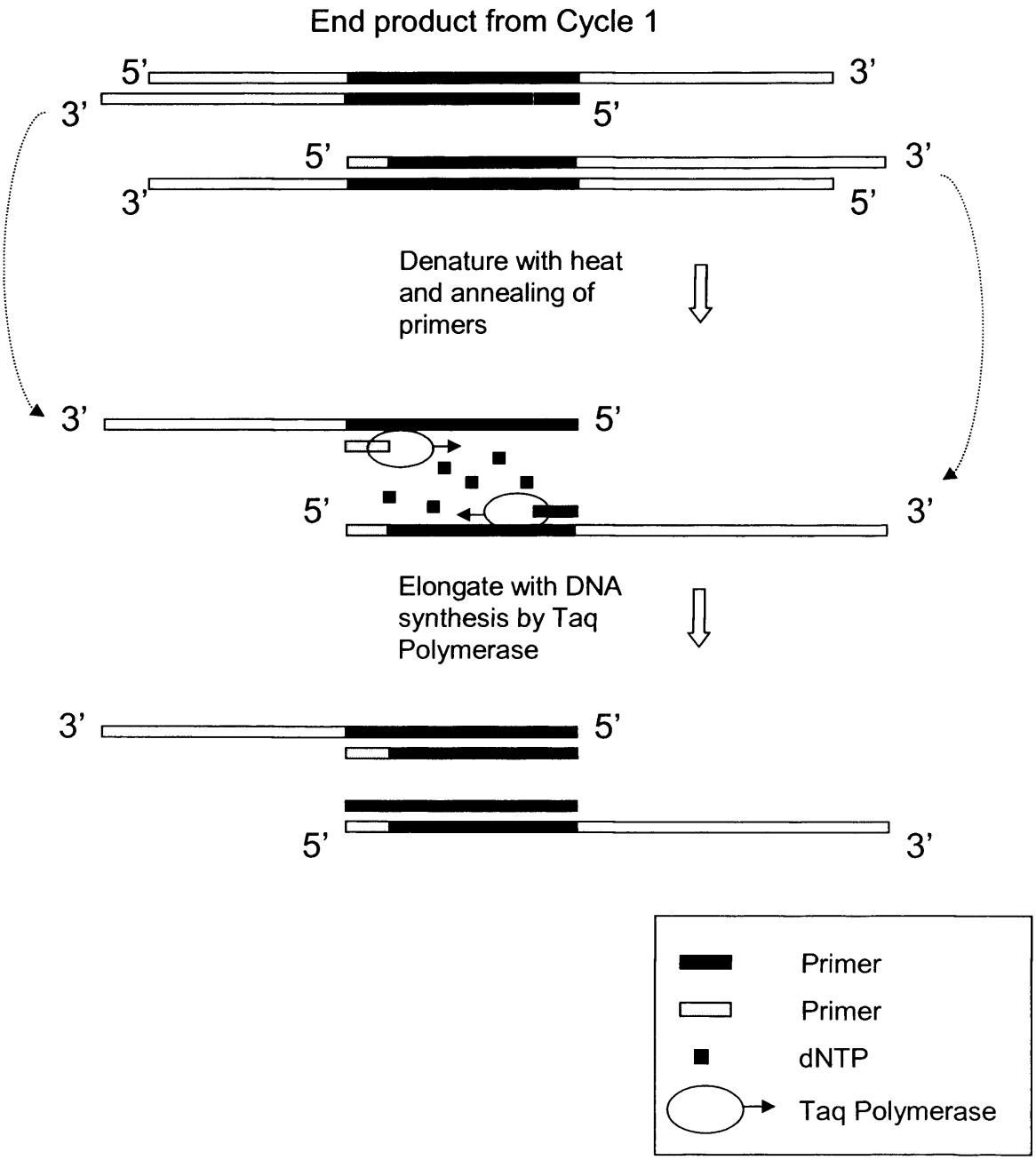


Figure 3.7 Cycle 2 of Polymerase Chain Reaction

The products from the end of Cycle 1 undergo a repeat round of denaturing, annealing and elongation.

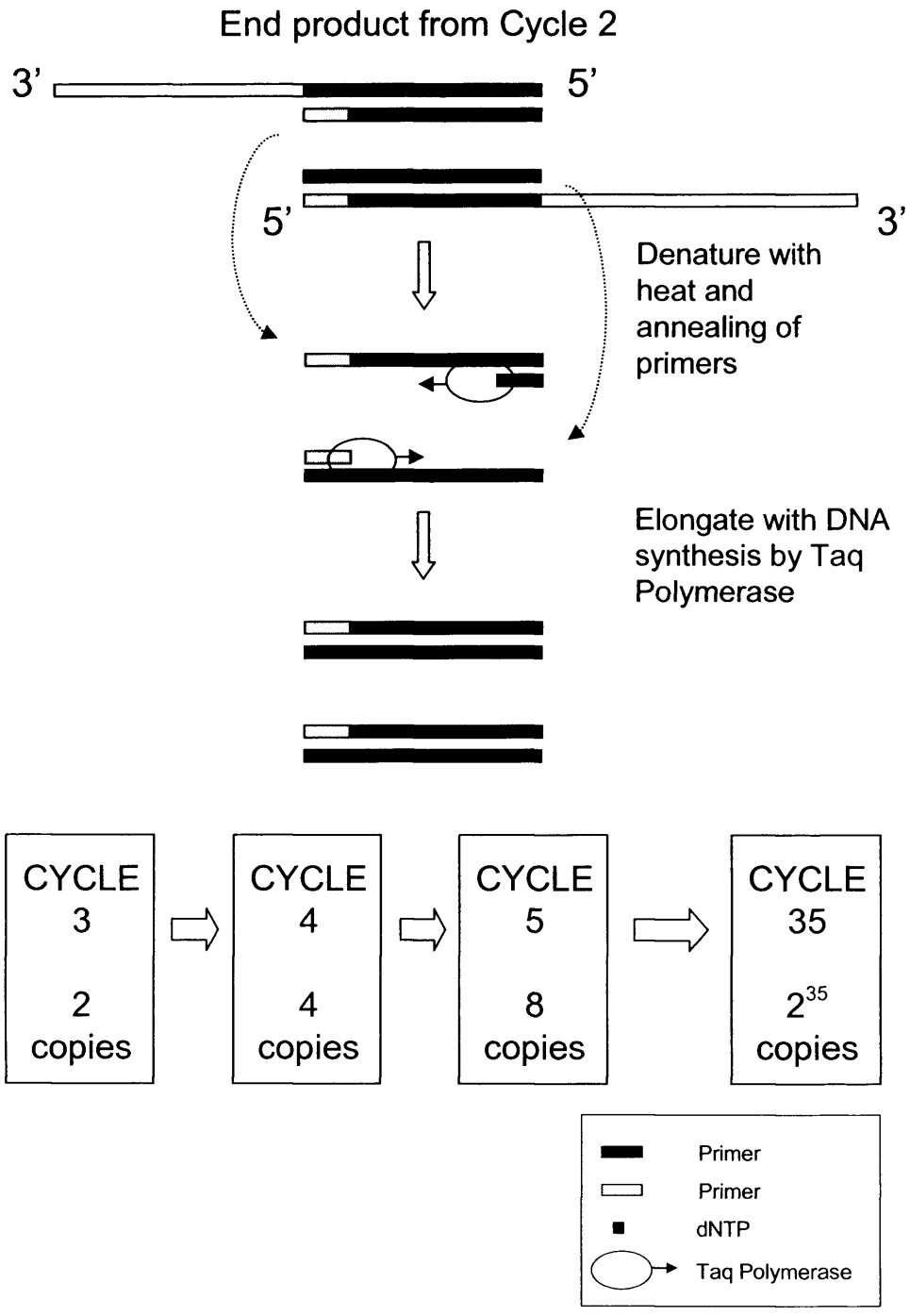


Figure 3.8 Cycle 3 of Polymerase Chain Reaction

Following a third round of denaturation, annealing and elongation, two copies of the target sequence of DNA have been produced. Thereafter, each cycle of the PCR will double this number; after 35 cycles, there will be 2^{35} copies.

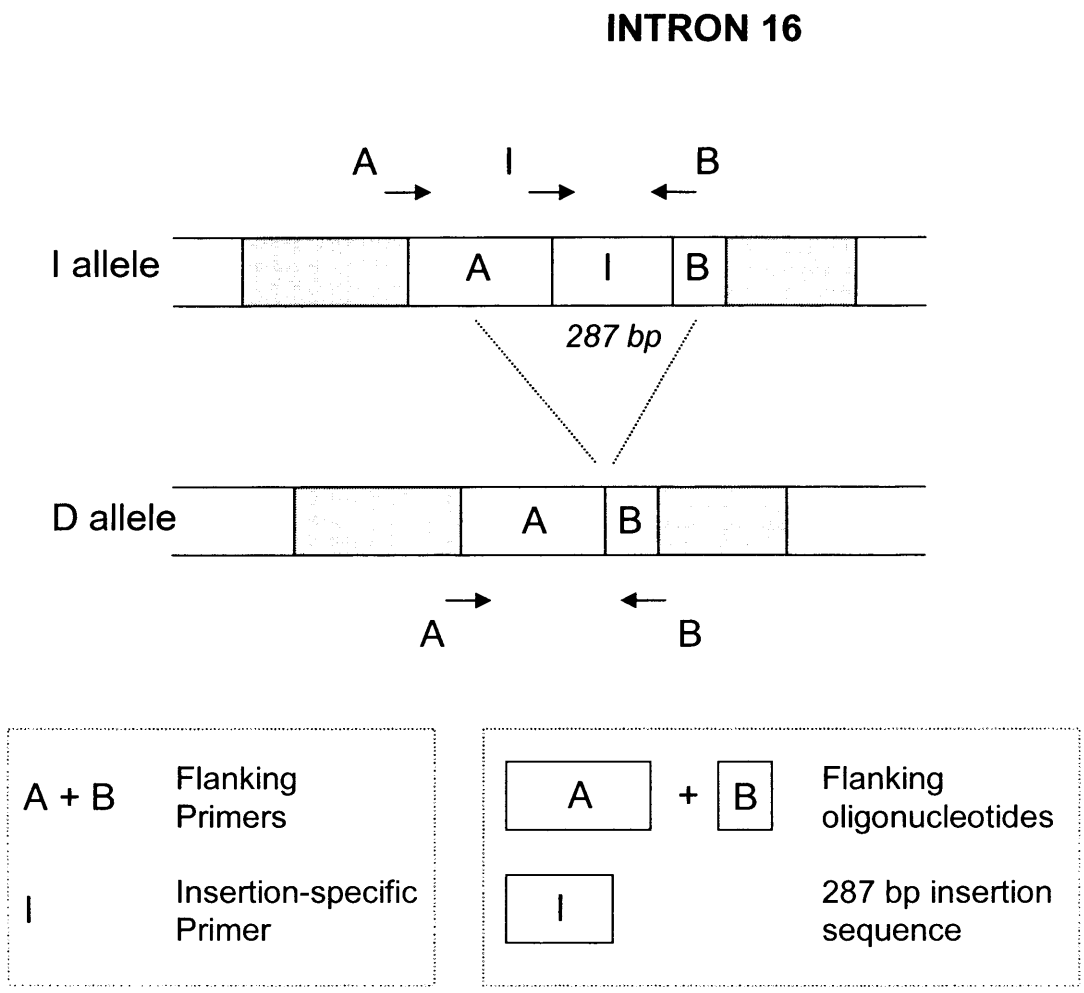


Figure 3.9 Representation of primers used to establish ACE genotype

Primers A + B were designed to amplify the sequence encompassing the potential site for the 287 bp insertion (corresponding to H ACE 3). An insertion-specific I primer (corresponding to H ACE 5) was designed to only recognise the I allele.

This relatively simple strategy for determining ACE genotype was first suggested by Rigat *et al,* (1992), but problems were subsequently reported with I/D heterozygote patients (Shanmugam *et al.,* 1993). These heterozygote patients will, by definition, possess one allele with the insertion sequence, and one without. Thus, PCR of the relevant stretch of their DNA sequence should yield two different sized products. However, cases of preferential amplification of the "deletion" rather than the "insertion" allele were described (Shanmugam *et al.,* 1993), whereby heterozygote patient samples were only yielding the smaller sized PCR product corresponding to the "deletion" allele. This led to inaccurate genotyping of patients as, in these cases, it was rendered impossible to distinguish between D/D patients and I/D patients.

Lindpaintner *et al,* (1995) overcame this obstacle to accuracy by employing two sets of optimised primers. The first set (named H ACE 3) were similar to primers used previously, and were designed to span the region of interest within intron 16, and amplify both "deletion" and "insertion" alleles, resulting in 319 bp and 597 bp PCR products respectively. Samples were then subjected to a second separate PCR, with an additional set of primers (H ACE 5) designed to only recognise a sequence specific to the "insertion" allele. This second reaction only yielded a product (335 bp long) in the presence of the "insertion" allele; there were consequently no products in D/D homozygotes. A graphic representation of this strategy is included in Figure 3.10.

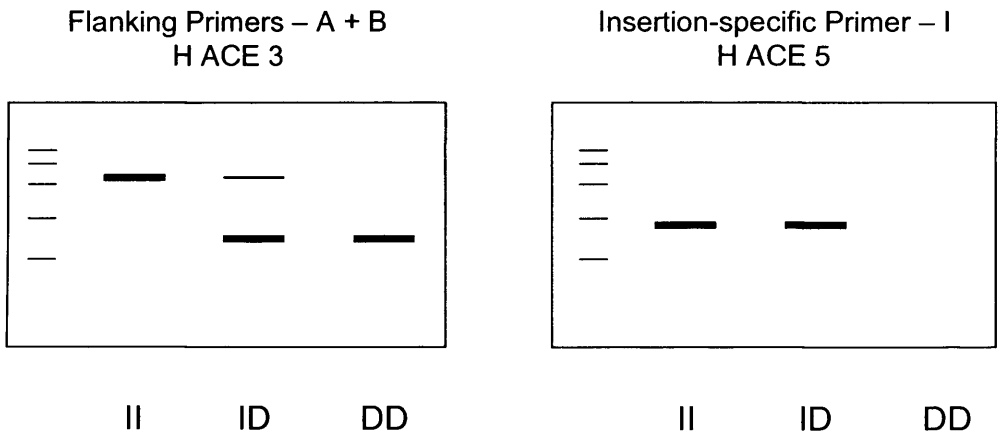


Figure 3.10 Illustration of PCR result to establish ACE genotype

An illustration as if products have undergone separation by agarose gel electrophoresis. Primers A + B produce different sized products depending on the presence of the I allele. Preferential amplification of the D allele leads to the need for a second, insertion-specific set of primers.

This same method for determining ACE genotype was employed in this study, and the two primer sets used are summarized below. The NCBI accession number for both the (F) and (R) H ACE 3 primers is *AY436362*, and for H ACE 5, it is *HSATICE* for the (F) primer and *AY436362* for the (R) primer.

Primer	SEQUENCE (5'? 3')	Tm (°C)	Binding Position (bp N°)	D Allele product (bp)	I Allele product (bp)
H ACE 3 (F)	-GCCCTGCAGGTGTCTGCAGCATGT-	74	13708 to 13731	319	597
H ACE 3 (R)	-GGATGGCTCTCCCCGCCTTGTCTC-	76	14019 to 13996		
H ACE 5 (F)	-TGGGACCACAGCGCCCGCCACTAC-	78	1590 to 1613	-	335
H ACE 5 (R)	-TCGCCAGCCCTCCCATGCCCATAA-	74	13997 to 13974		

The melting (or annealing) temperature of the primers (T_M) is defined as the temperature at which half of the primer will anneal to the target sequence, and the relevant values for the primers employed are included above. A good approximation of the melting temperature can be made by using the "Wallace formula":

$$T_M = [4(\text{Number of G} + \text{C}) + 2(\text{Number of A} + \text{T})]$$

As can be seen, a larger contribution to the melting temperature is made by G-C base pairs, and this is due to their greater stability secondary to increased hydrogen bonding.

3.4.4 Polymerase Chain Reaction – Reagents

The basic protocol for the PCR reaction was based on the recommendations of Sigma® (UK), the supplier for all of reagents used, unless stated otherwise. The individual

components of the PCR reaction, along with their relevance are listed below. In all cases they were stored at -20°C until required for use.

i) **PCR Buffer**

This contained 100mM Trizama®-HCL, pH 8.3, 500mM KCl, and was obtained as a 10 x concentration stock, and diluted to a final 1 x concentration with ultrapure water. The manufacturer's product information indicated that no DNase or RNase activity had been detected.

ii) **Magnesium Chloride ($MgCl_2$)**

This contained a 25mM solution. Magnesium acts as a co-enzyme and is required for DNA polymerase to work efficiently. The optimal concentration differs for each PCR, with higher levels resulting in a less stringent reaction and *visa versa*. Thus a key part of the optimization process involved varying the $MgCl_2$ concentration to produce a clean reaction without additional spurious products. This optimal concentration usually lies within the range of 1-2mM.

iii) **dNTPs**

This contained 10mM of dATP, dCTP, dGTP and dTTP. These nucleotide tri-phosphates are the building blocks of the PCR reaction, and are required by DNA polymerase for the synthesis of the sequence complementary to the DNA template.

iv) **Oligonucleotide Primers**

These were stored at a stock concentration of 100μM.

v) **REDTaq DNA Polymerase**

This specialized thermostable enzyme is isolated from the thermophillic bacterium *Thermus aquaticus*. It contained 1 unit DNA polymerase/µl in 20mM Tris-HCL, pH 8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Igepal CA-630, inert red dye and 50% glycerol. One unit is defined as sufficient to incorporate 10nmol of total dNTP into acid precipitable DNA in 30 minutes at 74°C. The presence of the inert red dye allowed quick recognition and confirmation of appropriate mixing of each reaction.

3.4.5 Polymerase Chain Reaction – Optimisation

The initial step to optimise the PCR reaction was to ensure as good a yield of clean DNA as possible from the "whole blood processing" and "nucleic acid extraction" protocols (Sections 3.2.3 and 3.3.2). A general consideration to eliminate the chance of contamination was the adoption of established "good laboratory practice" – involving the use of lab coats, gloves, and 70% ethanol to clean all surfaces. Furthermore, all pipette tips and eppendorf tubes employed were autoclaved at 120°C at 1.3 Bars for 22 minutes, using a Series 2100 autoclave (Prestige Medical, UK), with tape (3M, UK) used for the Bowie-Dick test to ensure sterility.

The stringency of a PCR reaction relates to the specificity with which the primers anneal to the nucleic acid template, and is directly proportional to the number of accurate base-pair matches between the primers and the target sequence. The lower the stringency, the less specific the annealing of primers and the greater likelihood of mis-matches between the primer and target sequence bases occurring. This will clearly come at the cost of a greater likelihood of sequences other than the planned target also

being amplified *ie.* a PCR that amplifies a similar but non-specific sequence, and is less "clean". Thus optimisation of the PCR involves altering the stringency to reach an ideal balance of specificity and sensitivity whereby adequate and accurate amplification of the target sequence occurs without the production of additional spurious products. This was achieved by altering the concentration of $MgCl_2$ within the reaction and the annealing temperature of the reaction; higher $MgCl_2$ concentrations and lower annealing temperatures produce lower stringency conditions.

The initial step was to choose an annealing temperature slightly lower than that predicted as the melting temperature by the Wallace formula, and combine this with higher $MgCl_2$ concentrations to produce less stringent conditions and allow more generic amplification of target sequences. This was followed by trials of increasing stringency by increasing the annealing temperature, or lowering the $MgCl_2$ concentration, until a "clean" (as in few additional non-specific bands) yet accurate PCR was obtained. Positive and negative controls were always used to ensure that this process of "fine tuning" was accurate.

The time lengths of each part of the cycle required fine-tuning during optimisation. The annealing time was set at 45 seconds, which was long enough for complete annealing, but not too long, so as to allow the amplification of non-specific products. Working at 72°C, Taq DNA Polymerase is able to elongate at an efficiency of around 1000 bp per minute. Given that the expected product sizes were well below 1000 bp, an elongation time of 60 seconds was used.

The amount of starting template was a final consideration when establishing a robust and effective protocol. The aim of this PCR reaction was to establish the presence and size of target sequences, with no need to make an analysis or comparison of the number of transcripts. Thus, it was not strictly necessary to have an identical amount of starting template of DNA for each reaction. Nevertheless a minimum of 100ng of DNA in 5µl was established as necessary.

3.4.6 Polymerase Chain Reaction - Protocols

In all cases, the reaction was set up on ice (4°C) to ensure consistency of ambient temperature. A "mastermix" was made up, consisting of ultrapure water, PCR Buffer, $MgCl_2$ and dNTPs, to ensure accurate volume aliquoting of these generic reaction components, at their required concentrations, into separate 0.5ml eppendorf tubes (see Table 3.1 for details). The aim of the "mastermix" was to try to eliminate pipetting errors, and to minimise the variation that is inherent when trying to deal with very small volumes. A 5µl volume, adjudged to contain 100ng of sample DNA template, was then added to each reaction tube. A DNA template that was known to produce the desired product was used as a positive control, and a 5µl volume of ultrapure water, containing no template, was employed as a negative control. Once all these components of the reaction had been added, tubes were vortexed for 5 seconds to ensure adequate mixing, and then pulse centrifuged at 2000 x g to bring all the contents of the tube to the base.

Each reaction tube was then placed in a Progene thermal cycler (Techne Ltd, UK) and subjected to an initial 4 mins at 94°C to ensure denaturation of the DNA template. Next, a "mastermix" of the REDTaq DNA polymerase was prepared (see Table 3.1 for details), with the enzyme in sufficient concentration to produce adequate amplification.

Reagent	Stock Conc	Final Conc	Volume added for n samples (µl)
PCR MASTERMIX			
PCR Buffer	10x	1x	4.5(n)
$MgCL_2$	25mM	1.5mM	3(n)
dNTPs	10mM	0.2mM	n
Primers (F+R)	100µM	1µM	n
Ultrapure H_2O			30.5(n)
DNA template			5
	Total in each reaction		45
Taq MASTERMIX			
PCR Buffer	10x	1x	0.5(n)
Taq	1u/µl	0.1u/µl	0.5(n)
Ultrapure H_2O			4(n)
	Total in each reaction		5
	Total PCR Reaction Volume		50

Table 3.1 PCR reagent concentrations and mastermix volumes - ACE

Summary of optimised PCR conditions for establishing ACE genotype.

5µl of this mix was added to each individual reaction, using a 65°C "hot start" to improve the efficiency of priming and amplification. This completed the addition of required reaction components, and so each tube could then proceed to undergo 35 rounds of thermal cycling. A final period of 10 minutes of polyadenylation at 72°C at the end of the cycling was included in the protocol, to ensure complete sequence extension. A summary of the PCR thermal cycling conditions is included in Table 3.2.

3.4.7 Analysis of Transcripts by Gel Electrophoresis

Once the PCR has ended, the products must then be analysed, and this is achieved by gel electrophoresis. This process involves using an electric field across a gel matrix to act as a form of "sieve", separating out molecules according to their size and charge. Given the same prevailing condition, the "frictional" force exerted by the gel matrix means that larger molecules will migrate for a shorter distance through the gel, and this forms the basis of resolving PCR products according to base pair length. Due to the large number of phosphate groups contained, DNA carries a uniformly negative charge, making it very amenable to separation by electrophoresis; it will migrate through an electric field towards the positive electrode.

The two main materials used to produce the gel matrix platform are agarose and polyacrylamide. Polyacrylamide gels consist of a finer matrix than agarose, and are consequently better at resolving DNA fragments by size, especially with smaller products below 100 bp. However, they are more difficult to produce and handle, and given the expected sizes of the PCR products (319, 335, 597 bps), the superior resolving power of polyacrylamide was not felt to be necessary in this study.

	Temperature (°C)	Time
Denature	94	4 mins
Hotstart	65	
Denature	94	45 secs
Anneal	64	45 secs
Elongate	72	60 secs
Number of Cycles = 35		
Polyadenylation	72	10 mins

Table 3.2 PCR cycle temperatures for ACE genotype

Summary of optimised temperatures and lengths for each cycle of the PCR for establishing ACE genotype.

Instead, agarose, a linear polysaccharide extracted from seaweed, was used to form a more robust gel matrix for electrophoresis. The greater the concentration of agarose within the gel, the finer the effective resulting matrix, and the better it is at resolving smaller product size. The agarose gel was made up using Tris-Borate-EDTA (TBE). As well as being a gel constituent, TBE was also used to immerse the gel within the gel rig, and thus act as a running buffer. Such a buffer was required to maintain a constant pH environment for PCR product migration, and to produce a conduit for the electric current flow.

Once the PCR products have been separated by gel electrophoresis, then some way is needed to visualise them. In this study, Ethidium Bromide was used to achieve this, exploiting its ability to intercalate with DNA to form a complex that can be visualised using an ultraviolet light transilluminator. Significant care was exercised when handling Ethidium Bromide, as this very ability to intercalate with DNA makes it potentially carcinogenic.

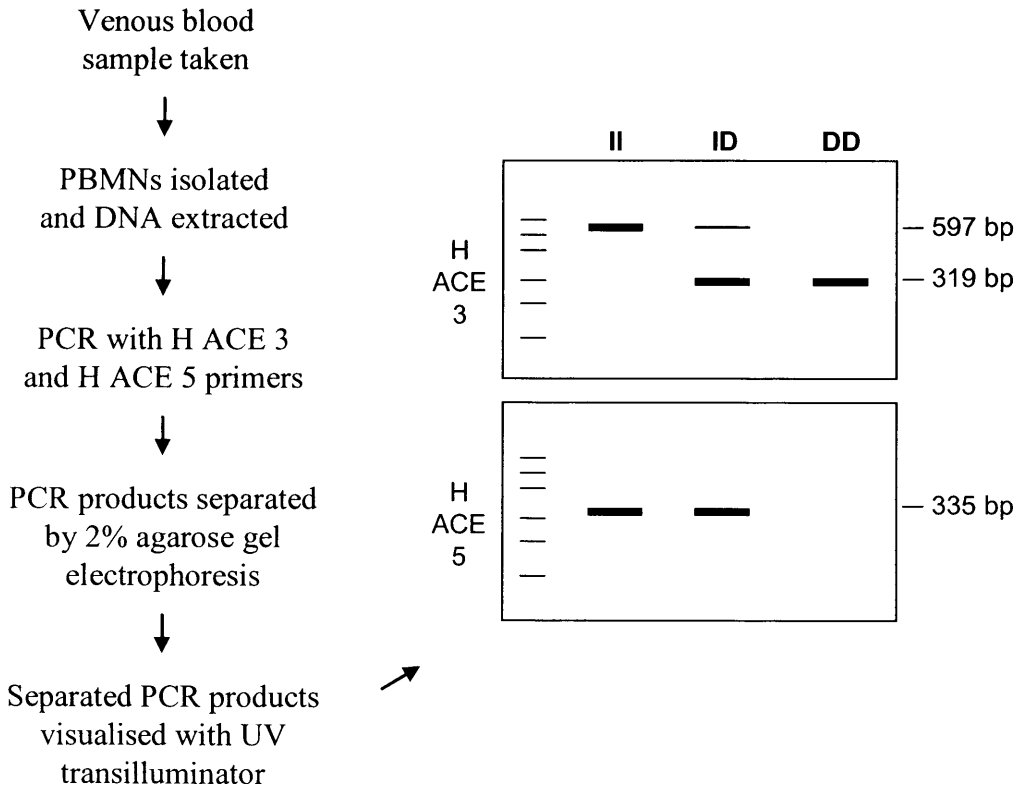
The protocol used is summarised in Table 3.3 – in all cases, reagents were supplied by Sigma®, UK. The volumes mentioned refer to the production of 100mls of a 2% agarose gel, which fitted with the available small gel rigs. A loading buffer (30% glycerol in water and 0.25% bromophenol blue) was used to aid visualisation of gel loading, and to provide sufficient density for the products to sink to the bottom of the wells within the gel. The product size was estimated by comparison with the migration of a 100 bp ladder (Sigma®, UK) that was also loaded into one of the wells.

Protocol employed for agarose gel electrophoresis of PCR products	
Step 1	2g of agarose was dissolved in 100mls of 1 x TBE buffer (89mM Tris-borate, 2mM EDTA, pH 8.3)
Step 2	Mixture heated in a microwave at 600W for 6 minutes, until the agarose was completely dissolved in the buffer
Step 3	10 µl of Ethidium Bromide (50µg/ml stock) was added to the mixture
Step 4	Mixture poured into a gel tray that had been prepared by sealing off each open end with heat-resistant tape, and gel combs applied to produce wells for subsequent loading of PCR products
Step 5	Once the gel has cooled and set (*c.* 30 minutes later), the tape was removed from the open ends, the gel tray placed into a gel rig and submersed in 1 x TBE buffer
Step 6	10µl of PCR product was mixed with 2µl of loading buffer, and 10µl of this subsequent mixture loaded into each well of the gel
Step 7	5µl of 100 bp PCR ladder was loaded into the first well of each row, to allow DNA fragment size determination
Step 8	Once all PCR product samples were loaded, they were run at 120V, 60mA for 30 minutes to produce adequate separation
Step 9	Once separation was complete, the gel was transferred to a UV-light transilluminator for visualisation
Step 10	A Polaroid™ photograph was taken with F-stop set at 8 and 0.5 secs exposure, for later reference

Table 3.3 Protocol for agarose gel electrophoresis separation

A successful PCR was established by looking at the control lanes; the presence of a band in the positive control, and the absence of any products in the negative control were accepted as indicative of success.

Overall, a summary of the processes involved in establishing the ACE I/D genotype is included below:



3.5 ESTABLISHING PATIENT IL6 -174 G/C GENOTYPE

3.5.1 Background

A functional single nucleotide polymorphism of the cytokine IL6 within the 5' flanking promoter region of the gene at position -174 was first described by a group analysing patients with the inflammatory condition of systemic-onset juvenile chronic arthritis (S-JCA) (Fishman *et al.*, 1998). The impetus for this research was the observation that S-JCA patients demonstrated a characteristic rise and fall pattern of serum IL6 levels, which paralleled their daily fever spikes. It was suggested that this pattern may be due to differences in the control of IL6 expression – thus prompting a search for functional polymorphisms in the promoter region of the gene. One of the results of this search was the -174 G/C polymorphism, and the methods employed to establish it will be discussed.

3.5.2 Polymerase Chain Reaction

The ability of the polymerase chain reaction to amplify a particular target DNA sequence to such an extent that the total amount of material can be resolved by gel electrophoresis, and visualised macroscopically, has been discussed in Section 3.4. The same strategy was employed in this study to amplify the region of the IL6 gene containing the G/C single nucleotide polymorphism (SNP) at position -174, using a method based on that described by DeMichele *et al,* (2003). The position -174 refers to the number of base pairs upstream from the transcription start point for the gene.

The reagents used in the PCR for determining IL6 genotype were identical to those described earlier for establishing ACE genotype. Furthermore, the process of

optimisation that was undertaken prior to instituting the final experimental protocol was the same; both reaction annealing temperature and $MgCl_2$ were both titrated to produce as clean a PCR as possible. However, the primers used to target the flanking 5' promoter region of the IL6 gene (NCBI accession number *M22111*.1) were obviously different, and are described below:

Primer	SEQUENCE (5'? 3')	Tm (°C)	Binding Position (bp N°)	D Allele product (bp)	I Allele product (bp)
IL6 G/C (F)	-ATGCCAAGTGCTGAGTCACTA-	74	890 to 910	305	305
IL6 G/C (R)	-TCGAGGGCAGAATGAGCCTC-	76	1193 to 1974		

The technical details of carrying out the PCR were identical to those previously described, and the final protocol for the PCR conditions is included below, along with concentrations and volumes of reagents used in Table 3.4.

	Temperature (°C)	Time
Denature	94	4 mins
Hotstart	65	
Denature	94	45 secs
Anneal	64	45 secs
Elongate	72	60 secs
Number of Cycles = 35		
Polyadenylation	72	10 mins

Reagent	Stock Conc	Final Conc	Volume added for n samples (µl)
PCR MASTERMIX			
PCR Buffer	10x	1x	4.5(n)
$MgCL_2$	25mM	1.5mM	3(n)
dNTPs	10mM	0.2mM	n
Primers (F+R)	100µM	1µM	n
Ultrapure H_2O			30.5(n)
DNA template			5
	Total in each reaction		45
Taq MASTERMIX			
PCR Buffer	10x	1x	0.5(n)
Taq	1u/µl	0.1u/µl	0.5(n)
Ultrapure H_2O			4(n)
	Total in each reaction		5
	Total PCR Reaction Volume		50

Table 3.4 PCR reagent concentrations and mastermix volumes – IL6

Summary of optimised PCR conditions for establishing IL6 genotype.

3.5.3 Restriction Enzyme Reaction – Background

Unlike the ACE I/D polymorphism which involves a 287 bp difference in size between the target sequences of the two different alleles, the IL6 SNP G- and C-alleles will be identical lengths. Thus, one cannot simply use PCR alone to amplify the sequence region of interest, and hope to distinguish between the two alleles by resolving them according to size alone with immediate gel electrophoresis of the PCR products. Instead, a more sophisticated strategy is required, one that is able to distinguish between two sequences which only differ by one single nucleotide.

This seemingly impossible task is achieved by exploiting the properties of restriction enzymes. These were first described in studies of the bacterium *Haemophilius Influenzae*, which was found to use specific enzymes to cut up the DNA from invading organisms such as viruses (Hamilton Smith *et al.*, 1970), and thus "restrict" the ability of the virus to replicate. These endonuclease enzymes essentially act to destroy foreign DNA molecules, and consequently exert a protective function – a form of "microbial immune system". However, they do not randomly slice a DNA sequence. Instead, they can be highly selective about the site that is chosen for cleavage, and it is this specificity that has made them such a valuable experimental tool. Restriction enzymes work by recognising short specific base pair sequences, and can be divided into two broad categories depending on their subsequent behaviour:

Type I — Cut the DNA strand at a random point far from the recognition sequence, with this lack of predictability making them of less practical use

Type II Cut the DNA strand at a defined position close to, or within the recognition sequence, and thus result in "restriction" fragments of predictable length

Although other types and classifications of restriction enzyme exist, they are of less practical importance and their discussion is beyond the scope of this thesis. All prokaryotic bacteria have been found to contain these restriction enzymes, and so thousands of different ones have been isolated. Despite the duplication of specificity amongst these, there are still over 250 different types of recognition-sequences that have been characterised.

If a restriction enzyme has a recognition sequence that contains the site of an SNP, then it becomes possible to distinguish between the two possible alleles. In the case of one SNP allele, it may be that the recognition site is present, and so the restriction enzyme proceeds to cleave the DNA strand in a predictable manner. However, the alternative SNP allele will obviously have a slightly altered sequence, which no longer possesses the enzyme's specific recognition site, and so no cleavage will occur. This presence or absence of cleavage will have a clear influence on the size and pattern of DNA fragments produced by each allele, and this will be evident following separation by gel electrophoresis.

Turning to the specifics of using a restriction enzyme to distinguish between the G- and C-alleles of the -174 G/C SNP, the starting point was to analyse the sequence surrounding this site, and this is depicted in Figure 3.11

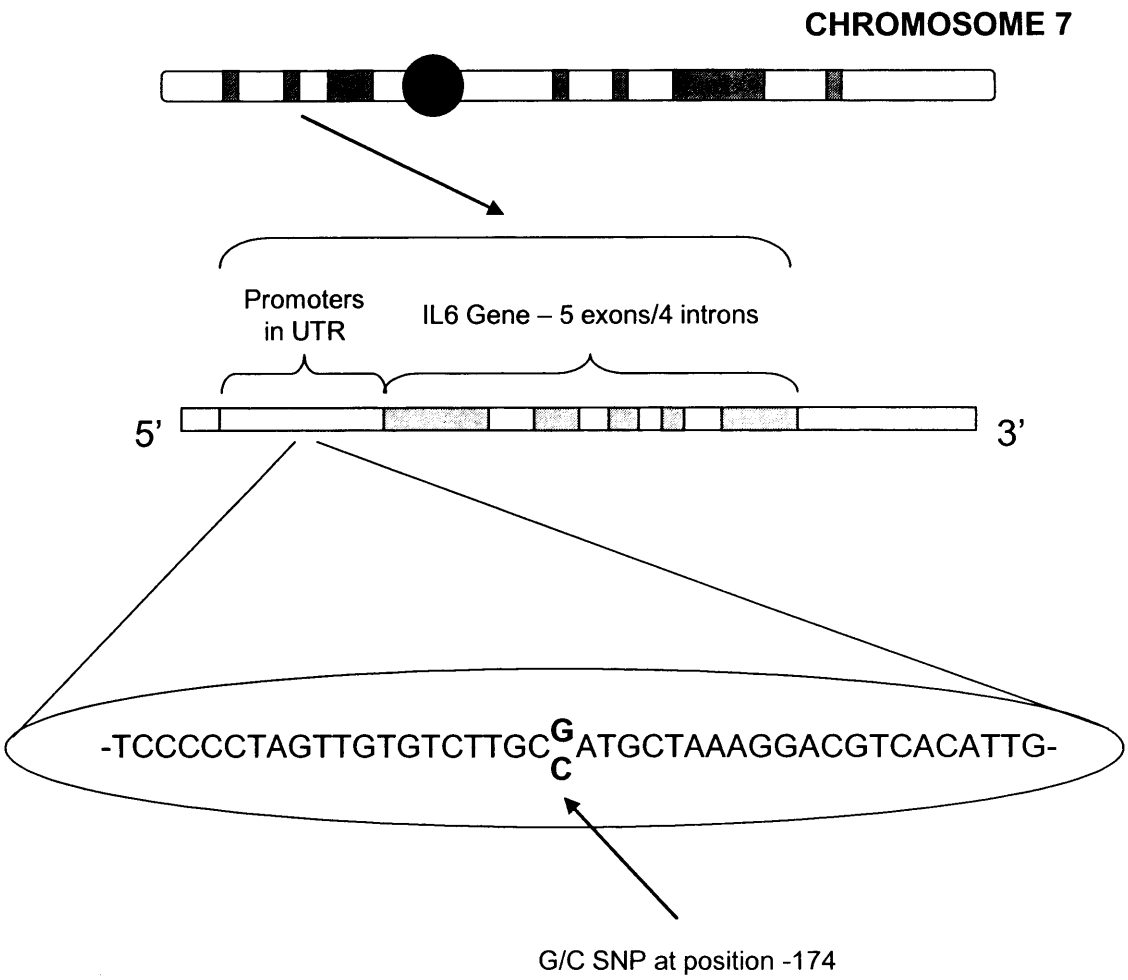
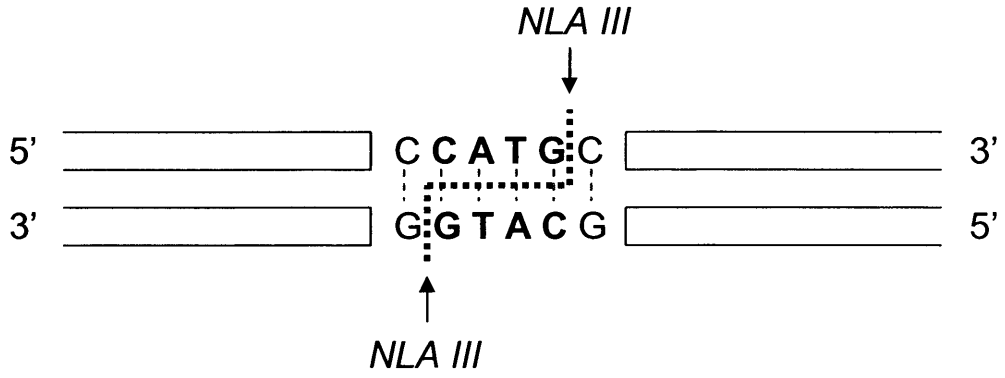


Figure 3.11 Representation of the site of -174G/C SNP of IL6 gene

The IL6 gene lies on the short arm of chromosome 7, and consists of 5 exons and 4 introns. The untranslated region (UTR) upstream from the gene contains the promoters which control expression. The G/C polymorphism lies within this UTR, 174 bp upstream from the transcription start site.

The key fact about the nucleotides surrounding the -174 SNP site is that they form part of a recognition sequence for the restriction enzyme *NLA III*. This enzyme has a target recognition sequence of "CATG". So, in the case of the C-allele, the NLA III enzyme's recognition sequence is intact, and cleavage of the DNA strand will occur, as illustrated below:



On the other hand, in the case of the G-allele, that recognition sequence is no longer present, and so the *NLA III* enzyme is impotent, and no DNA cleavage occurs. Thus we have the basis of the strategy employed to determine the -174 G/C SNP IL6 genotype:

Step 1	PCR to exponentially amplify the region of interest, containing the site of the SNP, so that it reaches macroscopically detectable levels
Step 2	Employ a Restriction Enzyme reaction, using *NLA III* that will only recognise the C-allele, to distinguish between alleles

In fact the sequence of the amplified PCR product contains another *NLA III* recognition site that is constant, not subject to an SNP, and distant to the -174 position. Thus, regardless of the genotype, an NLA III cleavage reaction will occur at least once, dividing the PCR product into a 230 bp and a 75 bp product.

3.5.4 Restriction Enzyme Reaction – Reagents

The basic protocol for the restriction enzyme was based on the manufacturer's recommendations (New England Biolabs, USA). In all cases, reagents were stored at -20°C until required for use, and are listed below:

i) **Enzyme Buffer (NE Buffer x4)**

Supplied as a 10x solution, and when diluted to 1x, consisted of 50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM dithiothreiotol

ii) **Bovine Serum Albumin (BSA)**

Supplied as a 100x solution

iii) ***NLA III* Restriction Enzyme**

This recognises a "CATG" oligonucleotide target sequence

3.5.5 Restriction Enzyme Reaction – Optimisation

Similarly to the PCR protocol, a period of optimisation was required for the Restriction Enzyme reaction. The optimal reaction buffer and BSA concentrations for *NLA III* enzyme function were based on the manufacturer's recommendations (New England Biolabs, USA). However, the two main factors that needed to be determined were:

i) The correct volume of PCR product (generated from the reaction described in Section 3.5.2) to add to the restriction reaction

ii) The amount of *NLA III* enzyme, and the length of incubation time

According to the manufacturer, 1 unit is defined as the amount of enzyme required to digest 1μg of DNA in 1 hour at 37°C, in a total reaction volume of 50μl. In addition,

the manufacturer's quality control assay reported that a 50µl reaction containing 1µg of DNA and 30 units of enzyme incubated for 16 hours resulted in the same pattern of DNA bands as a reaction incubated for 1 hour with 1 unit of enzyme. Thus, following a series of experiments to determine the optimal conditions for the restriction reaction, the following protocol was established; 30µl of PCR product, incubated with 10 units/reaction for 2 hours produced adequate cleavage (see Appendix I).

3.5.6 Restriction Enzyme Reaction – Protocol

Once again, all eppendorfs and pipette tips used were autoclaved to ensure sterility. Worktop surfaces were cleaned with 70% ethanol, and reactions set up on ice (4°C), to maintain cleanliness and consistency. For the same reasons as with the PCR, a "mastermix" was made up, consisting of ultrapure water, NE Buffer (x4), BSA and *NLA III* enzyme (see below for details).

Reagent	**Stock Conc**	**Final Conc**	**Vol/reaction (µl)**
NE Buffer x4	x10	x1	5
BSA	x100	x1	0.5
Enzyme	20,000U/ml	10U/reaction	0.5
Water	-	-	14
PCR Product			30
		Total Reaction Vol	50

20µl of the "mastermix" was aliquotted into suitably labelled 0.5ml eppendorfs on ice, and then 30µl of PCR product added to complete each reaction. Each reaction was then vortexed for 5 seconds to ensure adequate mixing, and then pulse centrifuged at 2000 x

g to bring all the contents of the tube to the base. Finally, the tubes were placed into a Progene thermal cycler (Techne Ltd, UK) and incubated at 37°C for 2 hours, before the reaction was terminated by denaturing the enzyme at 80°C for 20 minutes.

A summary of the strategy employed to determine the IL6 -174 G/C genotype is given below:

Venous blood sample taken

↓

PBMNs isolated and DNA extracted

↓

PCR with IL6 G/C primers

↓

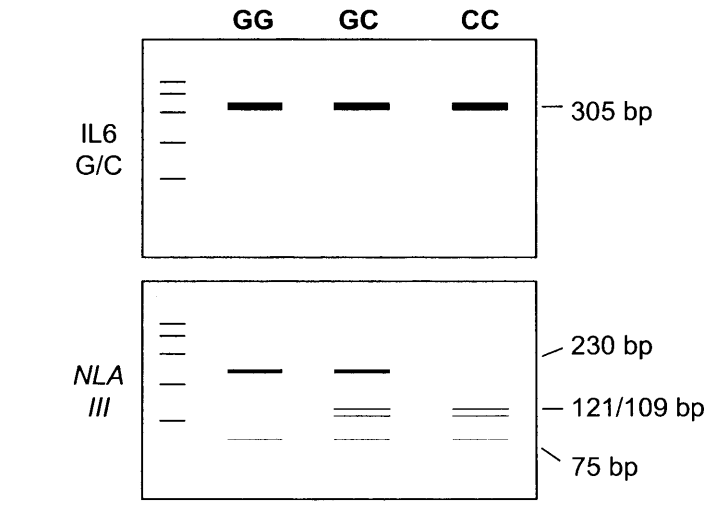
PCR products subject to *NLA III* restriction enzyme reaction

↓

PCR and restriction reaction products separated by 2% agarose gel electrophoresis

↓

Separated PCR products visualised with UV transilluminator



3.6 MOLECULAR ANALYSIS OF GENE TRANSCRIPTION

3.6.1 Overview of analysis of gene transcription

RNA represents the "final" nucleic acid product, providing the code for subsequent protein production by ribosomes. It differs fundamentally from the double-stranded DNA, in that it is single-stranded and has had the intronic "junk" sequences spliced out. Thus the single-stranded RNA is very unstable, but it can be converted into stable, double stranded complementary DNA (cDNA) by the process of Reverse Transcription (RT). This process requires an RNA-directed DNA polymerase, which uses the RNA as a template, to reverse transcribe a complementary strand of nucleotides, and thus produce the double stranded cDNA, as illustrated in Figure 3.12. In fact, this occurs naturally, when RNA viruses replicate within cells that they have infected.

The key point about the RT reaction is that the amount of resulting cDNA will be identical to the amount of starting RNA template. Thus, an assessment of the levels of RNA can be achieved by establishing the amount of cDNA present following an RT reaction. This can, in turn, be done by exploiting the fact that the double stranded cDNA can be amplified in the same way as DNA, using the polymerase chain reaction, as described previously. Thus, if samples are subjected to the same PCR, then they will have differing final amounts of cDNA at the end of the reactions, depending on the levels of starting RNA template; this will be evident macroscopically by the intensity of the bands produced following gel electrophoresis. Clearly there must be some form of control as well (discussed in Section 3.6.4), but nevertheless, this forms the basis for analysing gene transcription in a semi-quantitative manner.

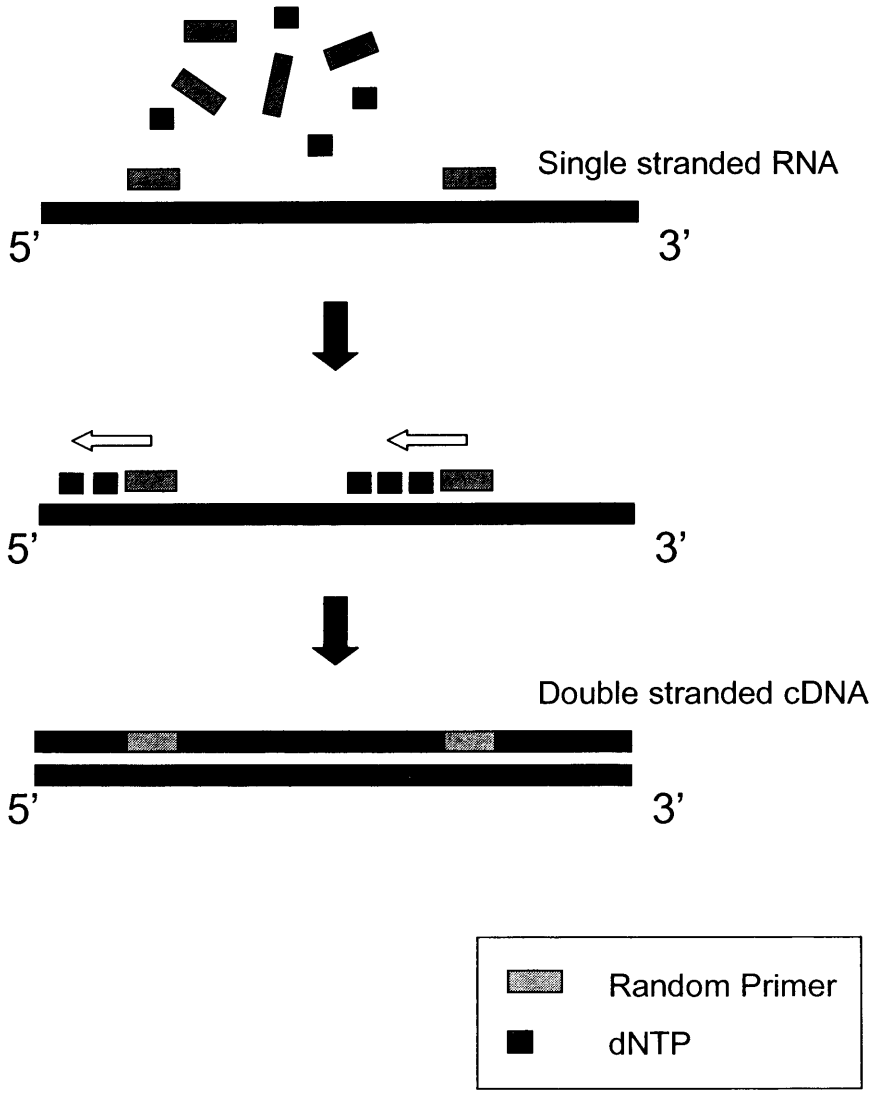


Figure 3.12 Reverse Transcription

Reverse Transcription is the process of converting unstable single-stranded RNA to stable double-stranded cDNA. dNTP, deoxynucleotide-triphosphate (dATP, dTTP, dCTP, dGTP).

3.6.2 Reverse Transcription – Reagents

All reagents were stored at -20°C until required for use. Ultrapure water used to make up the remainder of the reaction volume.

i) **RNA-dependent DNA Polymerase (MMLV)**

The enzyme used was derived from the Moloney Murine Leukaemia Virus (MMLV) (Sigma®, UK), and reverse transcribed single stranded RNA to produce double stranded cDNA. This additional strand synthesis is initiated by a primer, and involves the incorporation of the relevant nucleotides in a 5' to 3' direction. The supplied concentration was 200 units/µl; 1 unit being defined as enough to incorporate 1nmole of TTP into acid precipitable material in 10 minutes at 37°C (Houts *at al.*, 1979). It required its own buffer, with which it was supplied, to allow optimal functioning. This buffer contained 500mM Tris-HCL, 500mM KCl, 30mM $MgCl_2$, 50mM DTT.

ii) **Primers**

Nucleotide primers bind to their complementary target sequence on the RNA template, and are needed to initiate the function of the DNA polymerase enzyme. The nature of the primers will determine the specificity of the reaction. Random nonamer primers (Sigma®, UK) were used in this case, provided at a stock concentration of 50mM, with a recommended final concentration range of 1-4µM. These short random sequences of nine nucleotides bind complementary sequences at multiple sites of the template, and on all forms of RNA (message RNA, transfer RNA, ribosomal RNA) and thus result in the transcription of total cellular RNA. Alternative types of primer that were not used include OligodT

primers and gene specific primers. OligodT's consist of repeated thymidine nucleotides, which bind to the polyadenylation site at the 3' tail end of mRNA sequences, and are thus used specifically to bind that species of RNA. Gene specific primers have an even narrower spectrum of use; they will only reverse transcribe the segment of mRNA pertaining to a specific gene. These alternative primers were rejected because of the possibility of false negative results, and their greater expense.

iii) **dNTPs**

This contained 10mM of dATP, dCTP, dGTP and dTTP. These nucleotide tri-phosphates are the building blocks of the RT reaction, and are required by MMLV DNA Polymerase for the synthesis of the sequence complementary to the RNA template.

3.6.3 Reverse Transcription – Protocol

Once again, the basic protocol for the RT reaction was based on the product sheet provided by the MMLV DNA Polymerase's manufacturer's instructions (Sigma®, UK). As ever, surfaces were cleaned with 70% ethanol, reactions set up on ice (4°C), and all plasticware autoclaved prior to use.

Firstly, as it was important to know the final amount of cDNA produced, a volume of sample containing 2µg of template total RNA was added to pre-labelled, 0.5ml eppendorf reaction tubes. Next, 2µl of random primers and 2µl of dNTPs were added to each reaction, and the volume made up to 20µl with ultrapure water. Each tube was gently mixed and then pulse centrifuged at 2000 x g to bring all the contents to the

bottom before being placed into a Progene thermal cycler (Techne Ltd, UK). Samples were heat denatured at 70°C for 10 minutes, to eliminate any possible RNA tertiary structure, which may impair primer annealing. The samples were then removed and placed on ice for 5 minutes, to allow the random nonamer primers to anneal to the RNA template. During this period, a "mastermix" containing the MMLV DNA Polymerase enzyme within its buffer was made up (see below for details) and 20µl of this was subsequently added to each tube, to make a total reaction volume of 40µl and a final cDNA concentration of 50ng/µl.

Reagent	**Volume per Reaction (µl)**	**Mastermix Volume (µl) (n samples)**
MMLV enzyme	2	2(n)
MMLV buffer	4	4(n)
Water	14	14(n)
Total	20	20(n)

The tubes were then mixed and centrifuged before being placed back into the thermal cycler and incubated at:

i) First: 20°C for 10 minutes

ii) Then: 37°C for 50 minutes – to synthesise the cDNA

iii) Finally: 94°C for 10 minutes – to inactivate the heat-sensitive MMLV enzyme and thus end the reaction

Newly synthesized cDNA was stored at -20°C until required for use.

3.6.4 Polymerase Chain Reaction - Overview

The theory, basis and mechanism of the PCR have been described in Section 3.4, when discussing the amplification of DNA to enable the genotyping of patients. In this case of trying to analyse gene transcription, the reaction is seemingly identical, except for two major differences. Firstly, it is newly synthesized cDNA rather than normal DNA that is the starting material. Secondly, the final amount of cDNA at the end of the reaction is now of interest, as it is a measure of the amount of cDNA at the beginning of the reaction, and hence the level of RNA transcripts in the initial sample, and ultimately a barometer of target gene expression. For this to be at all meaningful, there must be the same amount of starting material added to each reaction, to make inter-sample comparison valid.

Moreover, there must also be some way to control for the general "activation level", in terms of the inflammatory or proliferative state of the cells being investigated. A cell that is, for either of these reasons, generally active, will produce a greater number of RNA transcripts for all genes. If this is not controlled for, then these cells may give the false impression of selective enhanced expression of a particular target gene, compared to other samples, when in fact this effect is not restricted but in fact global. Thus a "house-keeping" gene, which is constitutively expressed at a level that reflects the general "activation level" of that cell, is used as an internal standard control. Furthermore, such a method of normalisation also controls for experimental sampling errors that may also be responsible for spurious findings. So, transcription levels of a target gene are expressed as a ratio to the levels of the "house-keeping" gene, producing a semi-quantitative result for gene transcription within the sample. The "house-

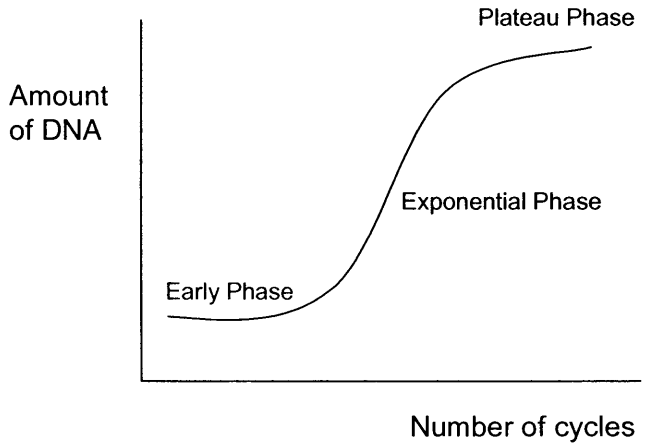
keeping" gene chosen for this study was glyceraldehyde-3-phosphate dehydrogenase (GAPDH -3).

However, this is not the only possible method of determining the levels of RNA present, and hence a measure of gene transcription. Northern blotting analysis is a 3-step process that remains the gold standard for splice-variant and size analysis (Bustin, 2005). Firstly, RNA is separated out according to charge and size by agarose gel electrophoresis, in a similar manner to that described in Section 3.4.7. The separated RNA bands are then transferred to a membrane, before a labelled probe is used to cross-link and hybridise with the RNA sequence of interest; the amount of probe binding to RNA gives a measure of the amount of RNA present. However, Northern blotting is a less sensitive method that requires larger amounts of starting RNA material for accurate results, and also becomes problematic when the RNA is degraded. Furthermore, if several target RNA sequences are of interest, then membranes must be repeatedly stripped and re-probed, which is an expensive and time-consuming process.

The RNAase protection assay can also quantify the amount of RNA present, and involves a radio-labelled oligonucleotide probe binding to the target RNA sequence. Next, a ribonuclease is used to degrade all remaining single-stranded RNA; the hybridized RNA sequence is protected from digestion and can be visualised by electrophoresis and autoradiography. This method can distinguish between related mRNAs that will migrate to similar positions during a Northern Blot, and may also successfully map intron/exon boundaries (Bustin, 2005). However, it does require a lot of starting RNA material to produce accurate results. *In-situ hybridization* is another method for assessing the levels of RNA present, and this complex technique is the only

way to localise transcripts to specific cells within a tissue (Bustin, 2005). Such an analysis was not of relevance in this study, as ACE transcription levels in systemic PBMNs rather than tissue was of interest.

A more recent advance in the measurement of RNA transcript levels has been the development of the Realtime RT-PCR technique. This highly sensitive method is related to the basic semi-quantitative RT-PCR protocol already described in Section 3.6.1, but is able to directly quantitate the PCR products as they are generated. A direct fluorescence system is often employed to achieve this – with a signal produced each time a primer anneals to the target sequence, and successful *Taq* Polymerase transcription occurs. As illustrated in the diagram of PCR-kinetics below, the extreme sensitivity of Realtime RT-PCR is due to its activity during the initial phase of the PCR reaction, where there are low levels of available template, and an abundance of reagents.



On the other hand, traditional RT-PCR is a one-off "end-point" method that hopefully occurs towards the end of the exponential linear phase, when duplication of the target sequence is still occurring, and before the plateau phase when the reaction slows due to the consumption of reagents.

However, the extreme sensitivity of Realtime RT-PCR means that small differences in primer efficiency will greatly affect the results. Thus, a large number of preliminary work-up experiments are required to determine the optimal primer and target sequence concentrations, so as to be able to make valid comparisons of transcription results. Moreover, the oligonucleotide primers themselves need to be specially designed and manufactured, and are consequently expensive to purchase. In addition the machinery required to carry out the Realtime method is also expensive.

Given all of this, the decision was made to employ the robust, sensitive, cost-effective and relatively straightforward and repeatable method of semi-quantitative RT-PCR to determine the relative levels of gene transcription in the study patients.

3.6.5 Polymerase Chain Reaction – Primers

As with the primers required for standard PCR, those employed in this case will determine the specificity of the reaction, and allow the targeting and amplification of a particular gene's cDNA. However, it may also be possible for the primers to inadvertently amplify any genomic DNA that may be present in the reaction, thus giving a false assessment of the level of gene transcription. This potential pitfall is combated by prudent primer design; the target sequence, for primer-annealing is chosen such that it crosses at least one intron-exon boundary. Thus, in theory, the primers will only recognise the RNA sequence which has had the intronic segments of the appropriate DNA sequence spliced out. More practically, if any contaminating genomic DNA is amplified, then it will result in a much larger sized product compared to the target RNA. Knowledge of the nucleotide sequence of the target genes, along with the

location of their introns and exons, allows such primer design to be possible. Other considerations that must be taken into account when designing primers include:

i) Primer Length

Both forward and reverse primers should be of similar length, and ideally around 18-24 nucleotides in length - too short, and problems with lack of specificity arise, but too long, and primer dimerisation becomes an issue.

ii) Primer Specificity

One must be sure that the target sequence chosen for primer annealing is unique to the gene chosen for investigation. Otherwise, the PCR may amplify sequences relating to a multitude of genes, making any result pertaining to the levels of transcription of the target gene invalid. This seemingly huge task has now been rendered fairly simple by the Basic Alignment Sequence Homology Tool (BLAST) that is freely available from NCBI on the internet (http://www.ncbi.nih.gov) – this will rapidly analyse the homology of the chosen primers, to ensure that there they will not inadvertently anneal to other parts of the genomic sequence.

iii) Melting Temperature (T_m)

The T_m has already been discussed previously in Section 3.4.

Bearing all these factors in mind, primers 20 bp in length were designed to assess both ACE and IL6 gene transcription, along with that of the "house-keeping gene" GAPDH-3.

These are summarised below. The NCBI accession number for the ACE primers was *J04144.1*, for the IL6 primers it was *BT019748* and finally for the GAP primers, it was *NM_002046.*

Primer	Binding Site	Sequence (5'? 3')	Binding position (bp N°)	T_m (°C)	Product size (bp)
ACE (F)	Exon 5	-CACCAATGACACGGAAAGTG-	1351 to 1370	64	216
ACE (R)	Intron 5	-GCATCAAAGTGGGTTTCGTT-	1566 to 1547	64	
IL6 (F)	Exon 1	-CACACAGACAGCCACTCACC-	125 to 144	65	139
IL6 (R)	Intron 2	-TTTTCTGCCAGTGCCTCTTT-	263 to 244	64	
GAP3 (F)	Exon 1	-GAGTCAACGGATTTGGTCGT-	123 to 142	64	185
GAP3 (R)	Intron 3	-GACAAGCTTCCCGTTCTCAG-	307 to 286	64	

3.6.6 Polymerase Chain Reaction - Optimisation

Having designed the primers, a period of optimisation was again required to establish the optimal PCR conditions in terms of $MgCl_2$ concentration and annealing temperature (see Appendix I). This process of titration to determine the ideal reaction stringency was no different to that described earlier in Section 3.4, and was required for both ACE and IL6 sets of primers. The amounts of starting cDNA template required for the different primer sets was found to be different – 200ng for IL6 and 400ng for ACE. However, the same PCR conditions were found to be optimal for both primer sets, which improved the efficiency of processing patient samples.

3.6.7 Polymerase Chain Reaction – Protocols

The same experimental protocol as described earlier in Section 3.4 was employed – in terms of reagents used and experimental methodology. Following the process of optimisation, the PCR conditions that were found to produce the necessary balance of stringency are outlined in Table 3.5.

Once the PCR was complete, the products were separated by gel electrophoresis in an identical manner to that described earlier in Section 3.4.7, and a Polaroid™ photograph taken. However, unlike conventional PCR, the result was not binary, dependent on the mere presence or absence of bands. Instead, an assessment of the intensity of each band was required, to give a result for the level of gene transcription. This was achieved by using scanning densitometry, which is discussed at length in Section 3.6.9.

3.6.8 Polymerase Chain Reaction – Problems

The major problem with PCR was the presence of contamination, manifesting as PCR product bands within the negative control. This was combated with scrupulous care to clean all surfaces, including gloved hands, with 70% ethanol, and carrying out all experimental work on foil paper. Much care was also taken with pipette technique, to ensure that nothing was drawn up into the actual barrel of the pipette, and that no cross-contamination between reaction tubes occurred. All reagents were kept as working solutions, which had been made up from the original tubes supplied by the manufacturers. By paying particular attention to all these details, the problem of contamination was eventually eliminated.

Reagent	Stock Conc	Final Conc	Volume added for n samples (µl)
PCR MASTERMIX			
PCR Buffer	10x	1x	4.5(n)
$MgCL_2$	25mM	1.5mM	3(n)
dNTPs	10mM	0.2mM	n
Primers (F+R)	100µM	1µM	n
Ultrapure H_2O			30.5(n)
DNA template			8
	Total in each reaction		45
Taq MASTERMIX			
PCR Buffer	10x	1x	0.5(n)
Taq	1u/µl	0.1u/µl	0.5(n)
Ultrapure H_2O			4(n)
	Total in each reaction		5
	Total PCR Reaction Volume		50

	Temperature (°C)	Time
Denature	94	4 mins
Hotstart	65	
Denature	94	45 secs
Anneal	64	45 secs
Elongate	72	60 secs
Number of Cycles = 35		
Polyadenylation	72	10 mins

Table 3.5 RT-PCR reagent concentrations and mastermix volumes

Summary of optimised RT-PCR conditions for analysing ACE and IL6 gene transcription.

3.6.9 Analysis of Results by Scanning Densitometry

Scanning densitometry uses a computer to make a quantitative measurement of the signal intensity from a digitalised image. In this case, the image was a photograph of an agarose gel, following electrophoresis separation of PCR products, which had been digitalised using an Epson GT-9500 scanner. In all cases, the same camera settings were used when taking the Polaroid image (exposure 0.5 secs, F-Stop 8), making results from different experiments as comparable as possible. Scanning densitometry works by assessing the shade of each pixel of the digital image, in the context of a greyscale of 256 shades that cover the spectrum between black and white. Thus differences between the intensity of different bands of PCR products can be measured based on the number of pixels within the bands, and their position within the greyscale range.

The software package used for this analysis was Labworks™ Image Acquisition and Analysis (Ultra-Violet Products Ltd, USA). Once the digitalised scan of the gel photograph had been imported into the program, lanes of 120 pixel widths were defined, corresponding to the lanes on the gel. This produced a lane-profile graph of image intensity, with peaks corresponding to the bands on the gel. Once this had been done, there were two main strategies for comparing band intensities.

One option was to evaluate the total area under the curve that forms the peak – the Integrated Optical Density (IOD). This method suffers from inconsistency, especially when the limits of the peak are ill-defined, and the experimenter is forced to be subjective about the boundaries for the IOD calculation.

An alternative, and much more objective strategy, was the measurement of the IOD solely at the point of maximal intensity within a band. This method requires minimal operator-input and proved to be far more consistent, repeatable and representative of the photos. A summary of these methods is illustrated in Figure 3.13.

Thus, Labworks™ (Ultra-Violet Products Ltd, USA) was used to calculate the IOD corresponding to only the peaks within the lane-profile graph, for all bands. This figure corresponded to the amount of cDNA present within the band, which was in turn a measure of gene transcription. A semi-quantitative analysis of this gene transcription was then performed by comparing the IOD of the band of interest with the IOD for the standard GAPDH-3 gene. Between experiments, the same settings and method of Labworks™ analysis was performed, to ensure validity of comparison.

A summary of the semi-quantitative RT-PCR strategy employed in the analysis of gene transcription is given in Figure 3.14.

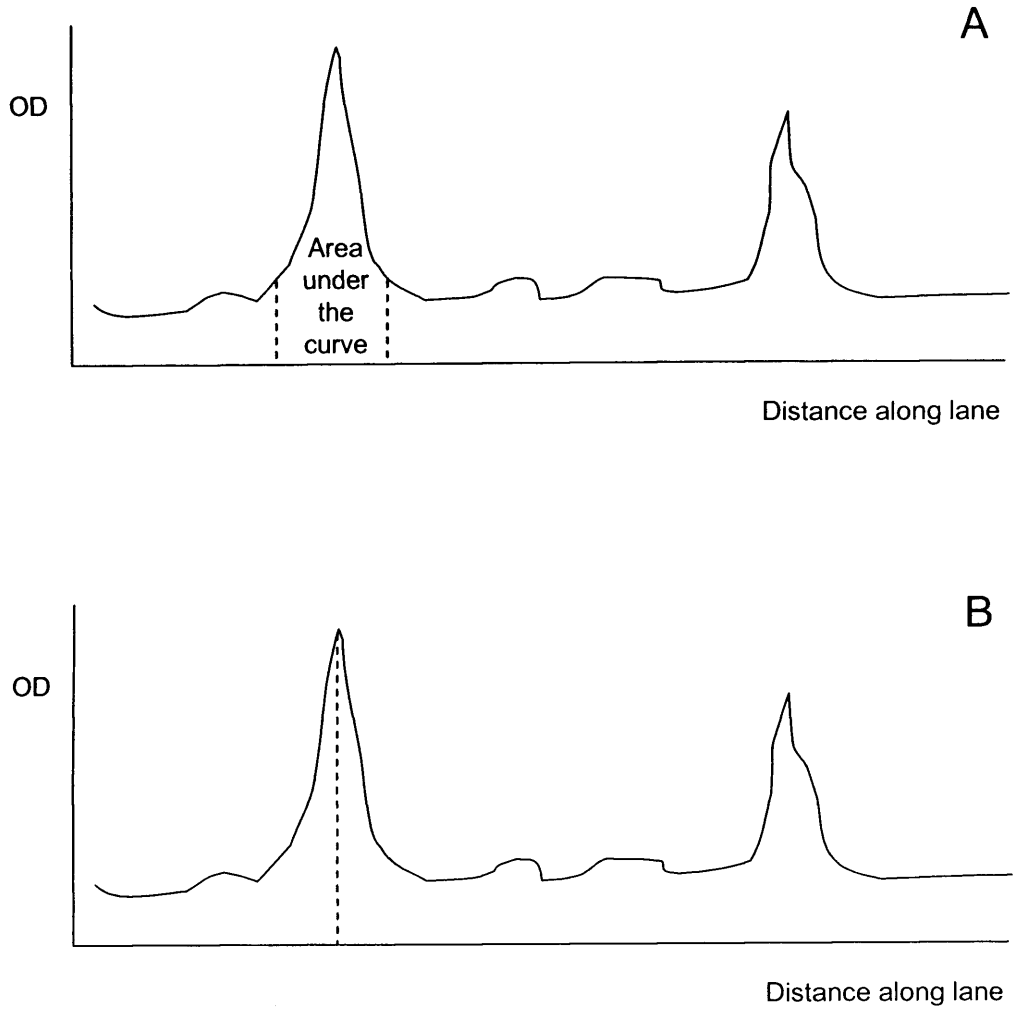


Figure 3.13 Methods of determining band intensity from lane-profiles

A, determining IOD for the whole peak – the area under the curve between the dashed lines. Placing the boundary lines is very subjective. **B,** determining IOD only at the maximal point of the peak. Placing the point for IOD determination is very objective. OD, optical density. IOD, integrated optical density.

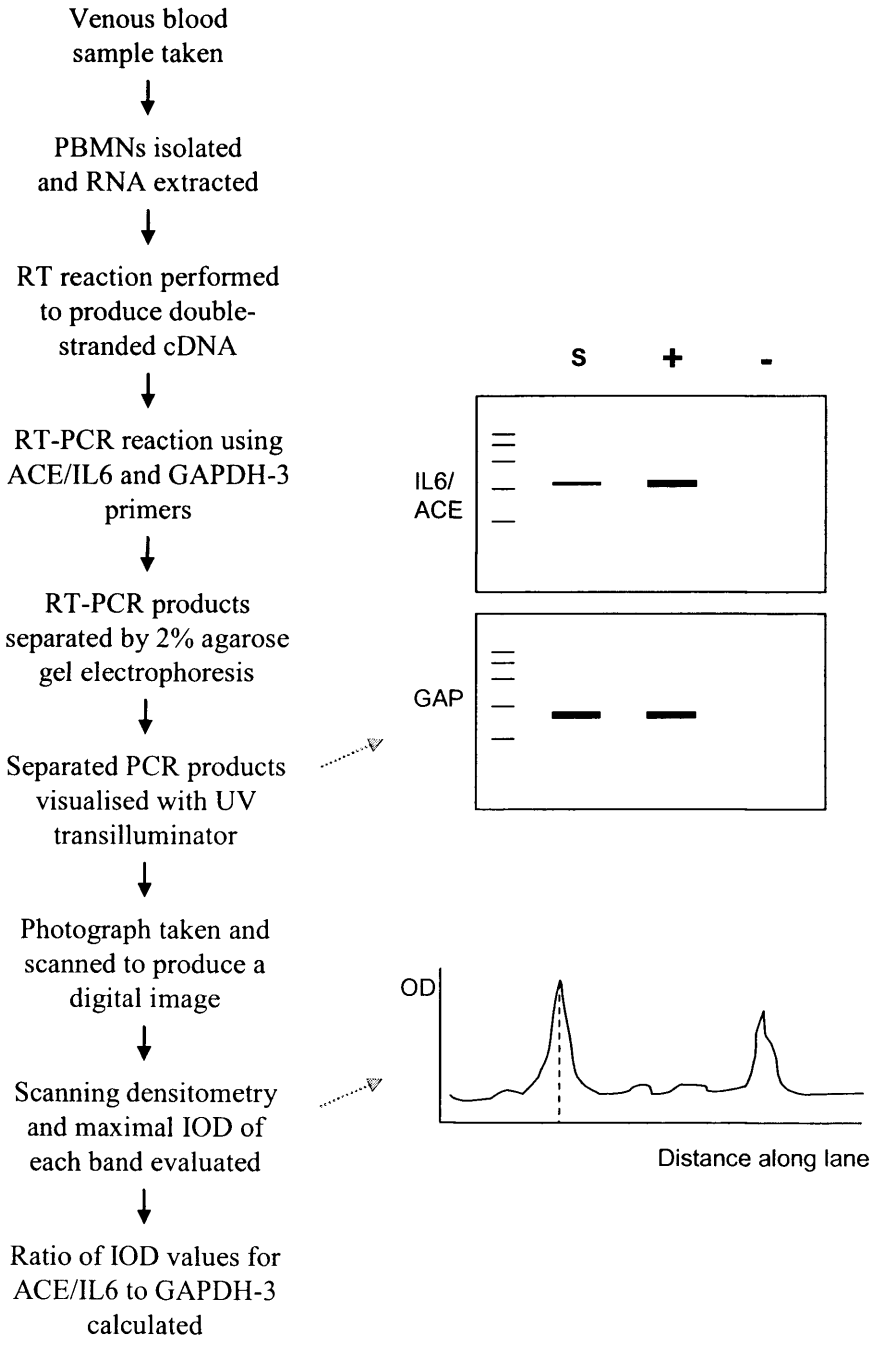


Figure 3.14 Summary of molecular analysis of gene transcription

Schematic flow-chart of the steps involved in the molecular analysis of gene transcription. RT, reverse transcription. cDNA, complementary DNA. PCR, polymerase chain reaction. S, patient sample. +, positive control. -, negative control. IOD, integrated optical density.

3.7 ANALYSIS OF DIFFERENTIAL TRANSCRIPTION OF ACE I/D ALLELES

3.7.1 Background

The ACE I/D polymorphism has received much attention with respect to its possible association with a plethora of disease states, mainly because it is known to be functional; as discussed in the Introduction (Chapter 1), the D allele is associated with high ACE levels. In an attempt to understand the potential mechanisms underlying this, the effect of the I/D polymorphism on gene transcription has been investigated in healthy volunteers (Suehiro *et al.,* 2004). This study examined the ACE mRNA expression originating from the D allele, with that from the I allele, in PBMNs from I/D heterozygotes. These heterozygotes provide the ideal focus for such an investigation, as both alleles are subject to identical cellular conditions and backgrounds, obviating the need for elaborate methods of experimental control.

The I/D polymorphism lies within intron 16, and is consequently spliced out, when the DNA sequence undergoes transcription into complementary RNA. Thus one cannot tell the allelic origin of an RNA sequence based on the I/D polymorphism alone. However, there exists a G/A SNP at position 2215 (G2215A), within exon 15, which leads to a silent mutation of the coding sequence, and is consequently transcribed into RNA (illustrated in Figure 3.15). So, by distinguishing between the two different RNA sequences, it is possible to determine which DNA allele an RNA strand originated from. Most importantly, the G2215A SNP is directly linked to the I/D polymorphism (Suehiro *et al.*, 2004), with every insertion allele linked to the 2215G allele.

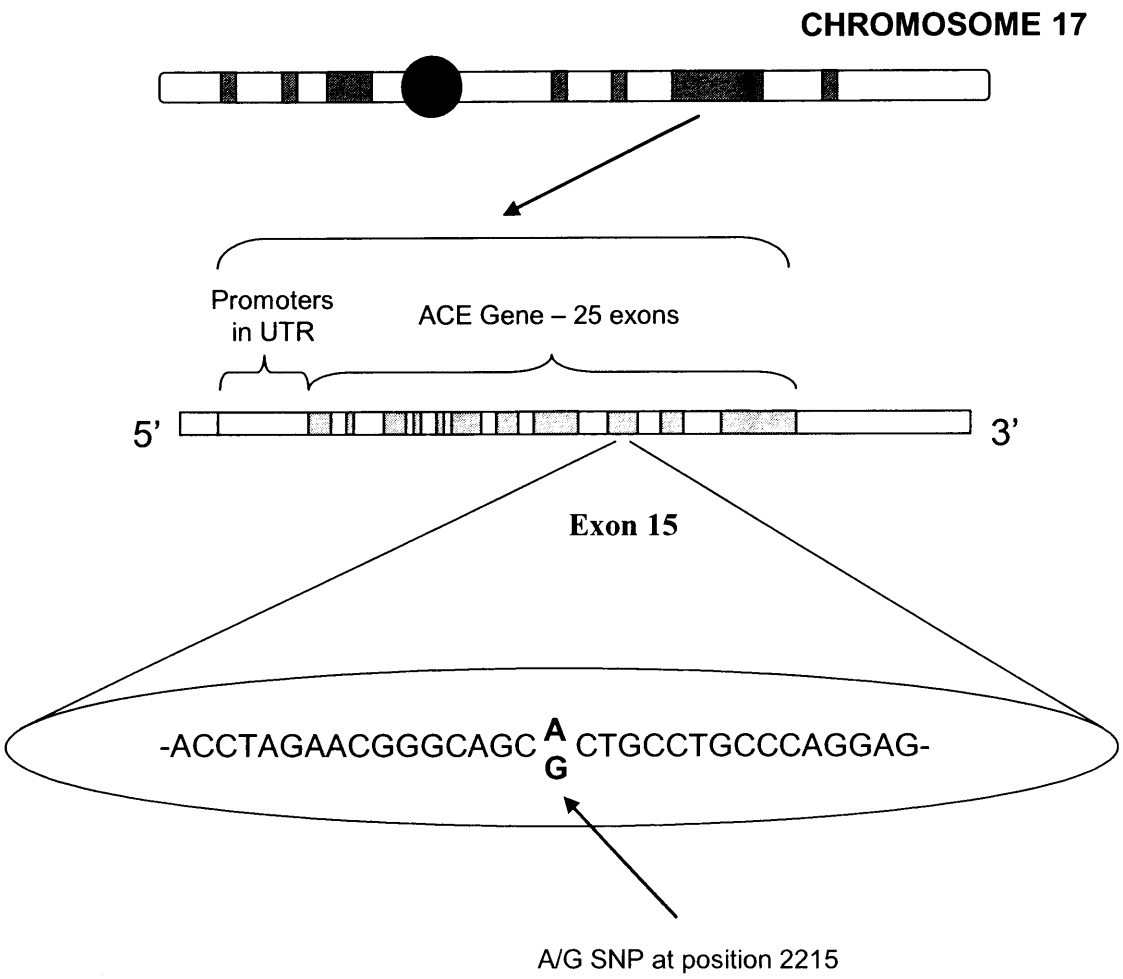


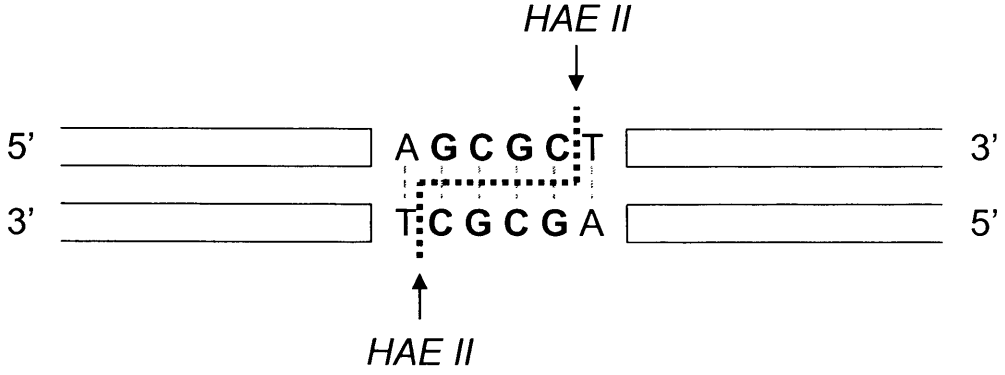
Figure 3.15 Representation of the site of A2215G SNP of ACE gene

The ACE gene lies on the long arm of chromosome 17, and contains of 25 exons. The A2215G polymorphism lies in exon 15, 2215bp downstream from the transcription start site.

Thus, ACE RNA with a G nucleotide at position 2215 must originate from the I allele, and this forms the basis of the method of comparing ACE allelic gene transcription.

The molecular basis of analysis of gene transcription has already been described in Section 3.6. An identical strategy of Reverse Transcription of RNA into cDNA, followed by a Polymerase Chain Reaction, will establish the overall level of ACE gene transcription. Careful choice of primers will achieve this by amplifying the target sequence region containing the G2215A polymorphism. Thus, if the amplified transcripts from the two different 2215 SNP alleles can be identified and separated, then this will effectively enable the comparison of the levels of gene transcription between I and D alleles.

Restriction enzymes have been described previously in Section 3.5.1, when discussing the IL6 -174 G/C genotyping. They can also be used with cDNA, and their ability to recognise and distinguish between short target nucleotide sequences again underlies the approach to differentiating between two alleles of an SNP. In this case of the G2215A polymorphism, analysis of the surrounding nucleotides demonstrates the SNP to lie within a recognition sequence for the restriction enzyme *HAE II*. This enzyme will only identify and cleave cDNA strands with the 2215G SNP, and ignore those with the 2215A, thereby allowing the two alleles to be distinguished. This is illustrated below:



The results of the *HAE II* restriction enzyme reaction will become evident following separation by gel electrophoresis (as described in Section 3.4.7). Of greater significance though, the relative intensity of the separated restriction digestion products will reflect the relative transcription originating from the G2215A alleles (and consequently the I/D alleles).

In summary, RT and subsequent PCR was carried out to establish overall levels of ACE gene transcription, and then *HAE II* digestion was performed on these amplified transcripts, using the silent G2215A exonic SNP to indirectly compare the contribution of the I and D alleles.

3.7.2 Primers and Reagents

The initial Reverse Transcription reaction to create stable cDNA from RNA was carried out in an identical manner, and using the same reagents, as described in Section 3.6.2.

For the subsequent PCR, the primers chosen to target the sequence encompassing the G2215A SNP were identical to those used in the initial study describing this technique (Suehiro *et al.*, 2004) and were supplied by New England Biolabs (US). They had the NCBI accession number *J04144.1* and are summarised below:

Primer	Binding Site	Sequence (5'? 3')	Binding position (bp N°)	T_m (°C)	Product size (bp)
ACE2215 (F)	Exon 15	-CACACCCTGAAGTACGGCAC-	2108 to 2127	65.5	242
ACE2215 (R)	Exon 17	-GTGGCCATCACATTCGTCAG-	2349 to 2330	66.6	

As can be seen, the primers were designed to target and amplify a 242 bp product. The rest of the reagents required for the PCR were identical to those described earlier in Section 3.4.4.

For the final step, the *HAE II* restriction enzyme (New England Biolabs, US) was supplied with its own buffer (NE Buffer x4) and Bovine Serum Albumin (BSA) – identical to the *NLA III* restriction enzyme described in Section 3.5.4. PCR products from the above reaction were incubated with *HAE II*. Those products originating from the 2215G allele, possessed the enzyme's recognition site (GCGC), and were cleaved to 153 bp and 89 bp fragments, whereas the products from the 2215A allele remained untouched. These fragments were separated by 2% agarose gel electrophoresis, using the same reagents and technique as described earlier in Section 3.4.7.

3.7.3 Optimisation

The two major steps of this protocol both required optimisation. For the PCR, this process was identical to that described earlier in Section 3.4.5; the amount of starting cDNA material, the concentration of $MgCl_2$ and the annealing temperature were all titrated to produce as clean a PCR reaction as possible.

The details of the *HAE II* restriction enzyme reaction also required fine-tuning, as described earlier in Section 3.5.5; the amount of enzyme needed, and the incubation period were the two variables to be determined. The enzyme's manufacturer's guidelines (New England Biolabs, US) formed the basis of the protocol, and stated that incubation with 1 unit of enzyme for 1 hour produced the same effect as with 30 units for 16 hours. With the previous restriction enzyme reaction (Section 3.5.5) the simple

presence or absence of cleavage was the only important result. However, in this case, with the relative intensity of the PCR products following cleavage being the key experimental result, it was essential to ensure as consistent and complete an enzyme reaction as possible. An initial experiment was conducted, looking at the degree of cleavage with different incubation times. The results are included in Appendix I, and the conclusion was that, for a reaction volume of 50µl (containing 30µl of PCR product) with 10 units of enzyme, incubation for 2 hours produced the same degree of cleavage as for 12 hours. Thus a protocol involving a 2 hour restriction reaction was established.

3.7.4 Final Protocols

The same experimental protocol as described earlier in Section 3.4 was employed – in terms of reagents used and experimental methodology. Following the process of optimisation, the PCR conditions that were found to produce the necessary balance of stringency are outlined in Table 3.6

Once the PCR was complete, a Restriction Enzyme reaction with *HAE II* was carried out, using the same protocol, methodology and conditions as described in Section 3.5.6. In summary, 30µl of PCR product was incubated with 0.5 units/reaction of the *HAE II*, for 2 hours at 37°C, followed by a 20 minute period of denaturation at 80°C to terminate the reaction. The fragments produced by the Restriction Enzyme reaction were separated by gel electrophoresis in an identical manner to that described earlier in Section 3.4.7, and a Polaroid™ photograph taken. An assessment of the relative intensity of resulting product bands was achieved by using scanning densitometry, as discussed in Section 3.6.9, and the process is summarised in Figure 3.16.

Reagent	Stock Conc	Final Conc	Volume added for n samples (µl)
PCR MASTERMIX			
PCR Buffer	10x	1x	4.5(n)
$MgCL_2$	25mM	2mM	3(n)
dNTPs	10mM	0.2mM	n
Primers (F+R)	100µM	1µM	n
Ultrapure H_2O			30.5(n)
DNA template			8
	Total in each reaction		45
Taq MASTERMIX			
PCR Buffer	10x	1x	0.5(n)
Taq	1u/µl	0.1u/µl	0.5(n)
Ultrapure H_2O			4(n)
	Total in each reaction		5
	Total PCR Reaction Volume		50

	Temperature (°C)	Time
Denature	94	4 mins
Hotstart	65	
Denature	94	45 secs
Anneal	64	45 secs
Elongate	72	60 secs
Number of Cycles = 35		
Polyadenylation	72	10 mins

Table 3.6 PCR reagent concentrations and mastermix volumes – ACE2215

Summary of optimised PCR conditions for analysing ACE allele differential gene transcription.

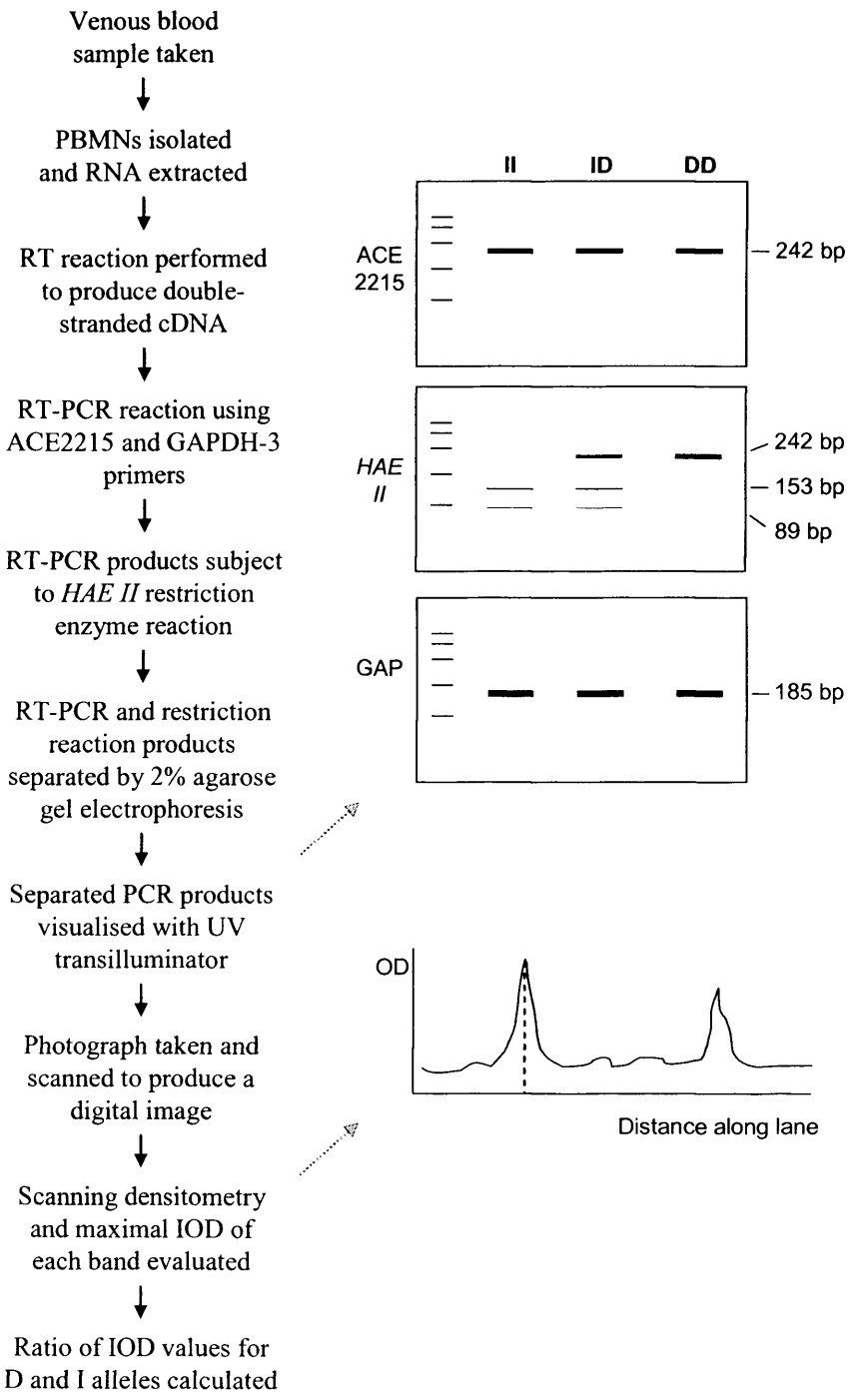


Figure 3.16 Summary of the method of establishing the differential allelic contribution to ACE gene transcription

Schematic flow-chart of the steps involved in establishing the allelic contribution to ACE gene transcription. IOD, integrated optical density. II/ID/DD patient results are depicted.

3.8 ANALYSIS OF PLASMA PROTEIN LEVELS

3.8.1 Background

There are two main methods available for the analysis of proteins that are soluble within the plasma – Western Blotting and Enzyme-Linked Immunosorbent Assay (ELISA). The technique of Western Blotting (Towbin *et al.*, 1979) evolved from the method of Southern Blotting; the identification of DNA sequences by gel electrophoresis separation, followed by binding of specific complementary probe sequences by base pairing. Instead of analysing DNA, a Western Blot employs the same principle for protein. To summarise the method in brief, the initial step is the separation of proteins according to size using polyacrylamide gel electrophoresis. Next, the separated molecules are transferred onto a separate membrane (usually nitrocellulose) and a labelled antibody is used as a probe to identify the target protein. Finally, some form of substrate is added as a detection method (*eg* colorimetric or fluorometric), with the intensity of the resulting signal correlating with the amount of protein antigen present. A key advantage of the Western Blot is the ability to visualise the protein and confirm its presence according to molecular weight. In addition, it is possible to strip and re-probe a single membrane for different proteins. However, in this case, this was outweighed by the complex multi-step nature of the protocol which would necessitate a great deal of optimisation. The experimental aim was not to confirm the presence of either ACE or IL6, but rather to establish their concentrations within the plasma. The decision was thus made to employ the other main method for soluble protein analysis – the ELISA.

3.8.2 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA technique is a relatively simple, robust and sensitive way of detecting and quantitating soluble proteins. It also exploits the highly specific interaction between antibody-antigen for the detection of proteins. There are two major types, the competitive and sandwich ELISAs, with the latter being particularly useful for the determination of antigen concentration in unknown samples; the exact application that was required.

The "sandwich" ELISA is so called because the protein antigen is bound between two different antibodies – "capture" and "detection". With this method, there is no need for initial protein separation and transfer. Instead, the first steps involve the immobilisation of antigens to a solid surface (usually the base of a 96-well plate), using a "capture" antibody. It is this binding and immobilisation that makes ELISAs easy to perform, as all non-specifically bound antigens can be washed away, meaning that the target protein is easily identifiable and measurable within a crude preparation such as plasma, which contains so many different proteins. A blocking protein is used to coat any exposed surfaces that do not have the capture antibody attached, thereby minimising any non-specific substrate binding.

Having been "captured" by one antibody, the next step is to "detect" the target protein using another antibody that recognises a different epitope on the protein surface, and completes the "sandwich". The assay is then quantitated by measuring the amount of bound "detection" antibody. There are several possible ways of achieving this, with the use of a colorimetric substrate being popular. This usually involves the "detection" antibody being biotinylated, and thus binding closely to a streptavidin-horse radish

peroxidase (strep-HRP) enzyme. Following the addition of a substrate, the HRP enzyme will catalyse a change in colour, from blue to yellow, the extent of which will correlate with the amount of protein present. The extent of this change can be assessed by using a plate-reader to measure optical density at 450nm, as this is the wavelength of the colour produced. A wavelength correction at 540nm is made for the potential effects of the plastic plate and reagents on absorption. Such an indirect method of measuring the presence of "detection" antibody may seem unnecessary – why not just label the "detection" antibody itself and identify that? Essentially, indirect detection prevents the chance of a bulky label affecting the immunoreactivity of the "detection" antibody. Furthermore, if each "detection" antibody contains several epitope sites that can be bound by an indirect enzyme, then signal amplification may occur, leading to enhanced sensitivity. These advantages outweigh the extra incubation step that must be added to produce an indirect detection protocol.

To make a quantitative assessment of this colorimetric change, a series of standards are run – normal experimental protocol is followed, except, instead of sample being added for assessment, known concentrations of target protein are added to a series of wells. This generates a standard curve of protein concentration against colorimetric response, from which it should be possible to calculate the levels of protein in each unknown sample. Figure 3.17 summarises this sandwich ELISA method.

Much of the success of a sandwich ELISA relies on the choice of the antibody pair for "capture" and "detection". The avidity with which they bind to the protein will determine in part the sensitivity of the assay.

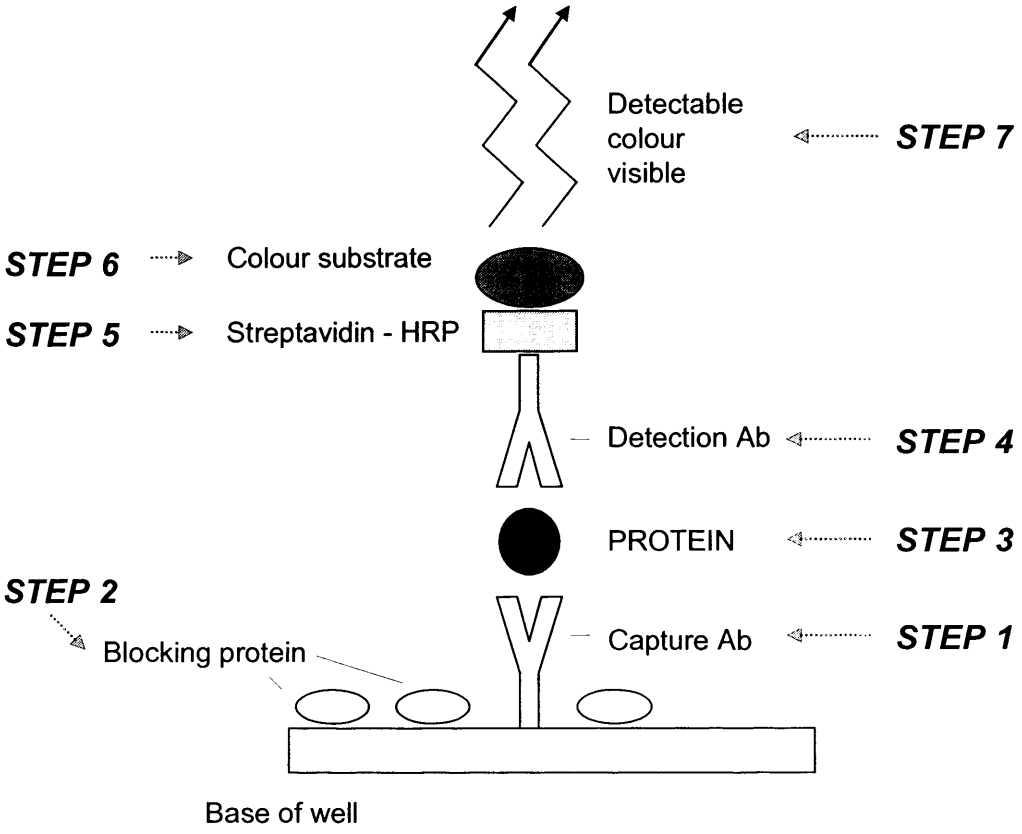


Figure 3.17 Schematic of Sandwich ELISA

The steps involved in performing a sandwich ELISA are illustrated, along with the final situation at the end of the assay.

However, they must also recognise separate epitopes on the protein surface, which are not so close together as to sterically hinder each other's binding. This is made easy by the availability of matched pairs within commercially accessible kits.

3.8.3 ELISA – Reagents and optimisation

All the reagents, unless otherwise stated, for the ELISA for both IL6 and ACE plasma protein detection were obtained as a "Duoset ELISA Development System" from the same manufacturer (R&D Systems, UK). The "capture" and "detection" antibodies obviously differed between ACE and IL6. However, the use of an indirect detection method with the sandwich ELISA meant that the same streptavidin-HRP and colour substrate could be used for both proteins. The basic protocol for the assay was taken from the manufacturer's recommendation (R&D Systems, UK). All reagents used were stored at 2-8°C until required for use, and are listed below:

i) **Phosphate Buffered Saline (PBS)**

Obtained from Sigma® (Sigma, UK) and consisting of 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM $KHPO_4$, pH 7.2-7.4 with solutions made up from new prior to each experiment

ii) **Reagent Diluent**

1% Bovine Serum Albumin (BSA) (Santa Cruz Biotech, USA) dissolved in PBS

iii) **Capture Antibody**

Goat anti-human ACE (Part N° 841365) – at stock concentration of 144µg/ml, and diluted to a working concentration of 0.8µg/ml, in PBS

Mouse anti-human IL6 (Part N° 840113) – at stock concentration of 360µg/ml, and diluted to a working concentration of 2µg/ml, in PBS

iv) **Detection Antibody**

Biotinylated goat anti-human ACE (Part N° 841366) – at stock concentration of 36μg/ml, and diluted to a working concentration of 200ng/ml, in Reagent Diluent

Biotinylated goat anti-human IL6 (Part N° 840114) – at stock concentration of 36μg/ml, and diluted to a working concentration of 200ng/ml, in Reagent Diluent

v) **Streptavidin-HRP**

Streptavidin conjugated to horseradish-peroxidase

vi) **Colour Substrate**

1:1 mixture of H_2O_2 and Tetramethylbenzidine

vii) **Stop Solution**

Used to stop the colorimetric detection assay – 2N H_2SO_4

viii) **Standard**

Recombinant human ACE at a stock concentration of 130ng/ml in Reagent Diluent – serial dilutions in Reagent Diluent made to produce a standard curve (range 8000pg/ml – 0pg/ml)

Recombinant human IL6 at a stock concentration of 45ng/ml in Reagent Diluent – serial dilutions in Reagent Diluent made to produce a standard curve (range 600pg/ml – 0pg/ml)

ix) **Wash Buffer**

0.05% TWEEN® 20 (Sigma®, USA) in PBS

x) **Sample Diluent**

Heat-inactivated Fetal Calf Serum (FCS) (First Link Ltd, UK), stored at -20°C, slowly thawed in a waterbath at 40°C when required and diluted in PBS

The aims of the optimisation process were to produce a robust, reproducible assay that was sensitive within the range of plasma concentrations that were expected to be encountered. Thus, the two main elements of the experimental protocol that could be titrated were the degree of dilution of patient samples, and the choice of diluent.

Clearly separate optimisation was required for the ACE and IL6 assays. Starting with the ACE ELISA, initial choices were for a 1 in 2 dilution of sample in 1% BSA. However, the results from these conditions proved unsatisfactory, as the colorimetric readings were all well above the upper end of the standard calibration curve, and thus unusable. Essentially, the sample concentration was too high, and so conditions with a greater degree of dilution (1 in 10 and 1 in 20) were tried. However, there was no improvement, with the same problem of excessively high colorimetric readings prevailing. The decision was then made to change the sample diluent to 10% FCS, and re-try a sample dilution of 1 in 20. This was again unsatisfactory for the same reasons, and so to provide a greater degree of "block", the diluent was changed to 20% FCS. In addition the degree of sample dilution was further increased to 1 in 40. These conditions produced readable and consistent results, and were used to establish the protocol below.

For the IL6 ELISA, 20% FCS was again chosen as the diluent, and a 1 in 2 sample dilution proved to be optimal in producing colourimetric readings that were within the boundaries of the standard curve.

3.8.4 ELISA - Protocols

The ELISA protocols were established after the period of optimisation. Assays were carried out in 96-well plates (Nunc™ Products, Denmark) and results read using a Plate-reader (Dynex Technologies Ltd, UK). All pipette tips and 1.5ml eppendorf tubes used, were autoclaved prior to use, as previously described. Each step was preceded by a wash step, involving complete filling of each well with wash buffer, using a squirt-bottle, followed by removal of liquid, repeated three times. Removal of wash buffer was concluded by inversion of the plate and blotting against clean paper towels. At all stages, thorough mixing of reagents was undertaken. Table 3.7 summarises the steps involved in an ELISA and the finalised protocols.

The only modifications of the protocol for IL6 were in Step 3 – the addition of sample or standard. For IL6, the dilution factor employed was 1 in 2, with 20% FCS as the diluent, and the standard was 600pg/ml as the maximal, with serial 1 in 2 dilutions again performed.

Protocol employed for sandwich ELISA for detection of plasma ACE			
	Procedure	Activity	Duration
Step 1	Capture coating	Capture antibody diluted to working concentration in PBS and 100µl added to each well. Plate sealed and left overnight in fridge at 2-6°C	Overnight
Step 2	Block	300µl of Reagent Diluent added to each well. Plate sealed and left on bench at room temperature	1¾ hrs
Step 3	Sample or standard	100µl of sample diluted by 1:40 in 20% FCS or standard (8000pg/ml top standard with serial 1:2 dilutions) added to each well. Plate sealed and left at room temperature	1¾ hrs
Step 4	Detection Ab	Detection antibody diluted to working concentration in Reagent Diluent and 100µl added to each well. Plate sealed and left at room temperature	1¾ hrs
Step 5	Indirect Detection	100µl of streptavidin-HRP added to each well. Plate sealed and left in the dark at room temperature	15 mins
Step 6	Colour Substrate	100µl of colour substrate added to each well – colour change should be evident. Plate sealed and left in the dark at room temperature	15 mins
Step 8	Stop	Without aspirating and washing, 50µl of Stop solution added to each well. Plate gently tapped to ensure complete mixing	
Step 9	Plate Reading	Plate placed inside plate reader, set to read at 450nm with 540nm correction	

Table 3.7 ELISA Protocol

The steps involved in performing a sandwich ELISA protocol are summarised, including the timings.

3.9 STATISTICAL ANALYSIS

All results were summarised using descriptive statistics. The Kolmogorov-Smirnov test for normality was used on all data, to determine the subsequent use of parametric or non-parametric tests when making group comparisons. Normally distributed data was presented as mean values with s.e.m. (in parenthesis), and multiple group comparisons made by one-way analysis of variance (ANOVA) with Bonferroni's correction to identify differences between individual group means. Alternatively Students paired or unpaired t-tests were employed as appropriate.

Data that was not normally distributed, was presented as median values with range (in parenthesis); multiple group comparisons were made using the Kruskal-Wallis test, and the Wilcoxon matched pairs or Mann-Whitney U tests were used as appropriate. Correlation between factors was analysed using Pearson's bivariate correlation analysis (parametric data) or Spearman's rank correlation test (non-parametric data). In all cases, the tests were two-tailed. From analyses, a value of $p<0.05$ was taken as statistically significant. Values of $p>0.5$ but <0.1 were deemed to be a trend, as reporting the 90% confidence interval.

CHAPTER 4

Patient Demographics and Outcomes

CHAPTER 4

4.1 INTRODUCTION

The outcome from major surgery is dependent on a combination of factors that are controllable (*eg* operative technique and post-operative care), and the patient's inherent ability to tolerate the operation. The importance of the latter has been recognised, with a multitude of strategies proposed and employed to identify "high risk" surgical patients: those whose management may need to be altered in view of their poorer ability to withstand the insult of major surgery (Lee *et al.,* 2006). Much of this focus has concentrated on patient physiology, and in particular, measures of functional capacity (Gerson *et al.,* 1990) and scoring systems such as ASA grade (Hall *et al.,* 1996). However, the individual immune response to surgery has received less attention, yet a greater understanding of the factors that influence its extent may aid surgical decision-making and provide potential novel therapeutic targets to improve outcome.

Surgery provokes an initial acute pro-inflammatory cytokine response (Sheeran *et al.,* 1997) as well as a later period of immunosuppression (Hensler et al 1997), the overall extent of which has been correlated with outcome following elective aortic surgery (Baigrie *et al.,* 1992). The wide variation in the reaction of the immune system to surgery may be partly due to genetic differences, and in particular functional cytokine gene polymorphisms (Damas *et al.,* 1997). These may in turn partially underlie observed inter-individual differences in outcome. Such a premise has been investigated for the functional -174 G/C polymorphism in aortic aneurysm (Bown *et al.,* 2003), organ transplant (Sankaran *et al.,* 1999) and cardiac bypass surgery (Gaudino *et al.,* 2003). Neither this nor immune responses have however been investigated in a more common surgical setting such as colorectal cancer surgery. Furthermore, there has only

been one study published looking at the influence of the functional I/D polymorphism of the ACE gene on surgical outcome (Lee *et al.,* 2005). ACE is increasingly thought of as multifunctional and pro-inflammatory (Cheng *et al.,* 2005), making its investigation in the peri-operative period a reasonable aim. Given the fact that colorectal adenocarcinoma is a common disease (CRC Cancer Statistics, 2003), which still has surgical excision as the gold standard for treatment (Rossi *et al.,* 2006), it provides a suitable cohort of patients for the investigation of the influence of IL6 and ACE polymorphisms on outcome.

4.2 MATERIALS AND METHODS

Ethical approval was obtained and elective patients recruited prior to laparotomy for colorectal cancer (see Section 3.1). A prospective record of post-operative complications and length of stay was made, and Dukes' stage taken from the histopathology report pertaining to the surgically excised specimen. White cell count and CRP levels were obtained from standard clinical laboratory tests. Blood samples were taken pre-operatively and, when possible, 4 hours, 24 hours, 10 weeks and 20 weeks following surgery. PBMNs were separated from blood by a process of centrifugation through a density gradient polymer and nucleic acids extracted using a phenol-ethanol precipitation method (see Sections 3.2 and 3.3). From isolated DNA, ACE and IL6 genotype were established by PCR (see Section 3.4).

4.3 RESULTS

Thirty one patients were initially recruited, although 2 were subsequently excluded (71 year old male had synchronous groin dissection for penile cancer; 34 year old male had a histological diagnosis of carcinoid). The remaining 29 patients (median age 69 (range, 43-87) years) consisted of 17 males (73 (43-87 years) and 12 females (69 (55-81) years), and were all Caucasian (see Appendix II and Table 4.1). Despite a slight preponderance of males within the study cohort, there was no significant difference in gender frequency ($p=0.353$, Chi^2; Table 4.1). Every patient underwent elective laparotomy for suspected or previously diagnosed colorectal adenocarcinoma. No patient was taking, or prescribed whilst in hospital, any form of corticosteroid or ACE inhibitor medication.

Pre-operative state of patient cohort

The majority of patients were ASA score II (79%; $p<0.001$, Chi^2; Table 4.1), with fewer ASA I (7%) and ASA III (14%). There was no difference in ASA score with gender ($p=0.33$, Chi^2). The mean pre-operative haemoglobin level was 12.1g/dl (±0.3, s.e.m.), with again no difference between males and females (12.1 vs 12.1g/dl, $p=0.99$, unpaired t-test; Table 4.1).

The most common disease stage was Dukes' C (41%), although this was not significantly greater ($p=0.52$, Chi^2) than either Dukes' A (24%) or B (34%) (Table 4.1). Anterior resection was the most frequently performed procedure within this cohort of patients (34%; $p<0.04$, Chi^2; Table 4.1). A summary of the specific operations undergone is given in Table 4.1.

	Male	Female	Total
Number	17	12	29
Age (years)	73 (43-87)	69 (55-81)	69 (43-87)
Operation time (mins)	210 (101-471)	250 (175-316)	227 (101-471)
Operation			
Right hemicolectomy	4 (57%)	3 (43%)	7 (24%)
Left hemicolectomy	2 (33%)	4 (67%)	6 (21%)
Sigmoid colectomy	1 (50%)	1 (50%)	2 (7%)
Subtotal colectomy	1 (50%)	1 (50%)	2 (7%)
Anterior resection	7 (70%)	3 (30%)	10 (34%)†
AP-resection	2 (100%)	0 (-)	2 (7%)
Hb (g/dl)	12.1 (±0.4)	12.1 (±0.4)	12.1 (±0.3)
WCC (x10^9)	7.9 (±0.6)	7.4 (±0.4)	7.7 (±0.4)
ASA			
I	2 (100%)	0 (-)	2 (7%)
II	12 (52%)	11 (48%)	23 (79%)*
III	3 (75%)	1 (25%)	4 (14%)
Dukes' Stage			
A	4 (57%)	3 (43%)	7 (24%)
B	5 (50%)	5 (50%)	10 (34%)
C	8 (67%)	4 (33%)	12 (41%)

Table 4.1 Demographic, operative and pathology details

Age and operation time values given as median (range). Hb, haemaglobin; WCC, white cell count; both given as mean (±s.e.m.). Percentages in parenthesis represent percentage/gender per operation type, ASA score and Dukes' stage. Values of significance; $^{\dagger}p<0.04$ and $*p<0.001$, both by Chi^2 test.

There was no significant difference between the sexes in terms of operation undergone ($p=0.6$, Chi^2), ASA score ($p=0.33$, Chi^2) and Dukes' stage ($p=0.73$, Chi^2; all in Table 4.1). Similarly, the overall operation time was median 227 (range, 101-471) minutes, with no significant difference ($p=0.49$, Mann-Whitney U) between males (210 (101-471) minutes) and females (250 (175-316) minutes; Table 4.1).

Peri-operative period

A measure of the effect of colorectal cancer surgery on the immune system can be gained from comparing pre- and post-operative white cell count (WCC) and C-Reactive Protein (CRP) levels. Results for WCC passed the Kolmogorov-Smirnov test for normality, prompting the presentation of data as mean ± s.e.m. and the use of parametric statistical tests. Results for CRP failed the Kolmogorov-Smirnov test for normality, so results are presented as median (range), and non-parametric tests were used to compare groups. At the pre-operative baseline timepoint, there was a significant correlation between CRP and WCC results ($p=0.001$, Figure 4.1), which was no longer evident post-operatively ($p=0.748$, both Spearman's rank correlation). There was a significant rise in WCC at 24 hours following surgery (7.7 ± 0.4 *vs* 10.4 ± 0.5 $x10^9$, Pre-op *vs* Post-op, $p<0.001$, paired t-test; Table 4.2A), as illustrated in Figure 4.2. Similarly CRP was also found to be significantly elevated at 24 hours post-operation (2.1 (0.4-271.3) *vs* 133.4 (16.4-279.2) mg/l, Pre-op *vs* Post-op, $p<0.001$, Wilcoxon Pairs; Table 4.2A) (See Figure 4.3).

Post-operative outcome

Moving on to outcome, there were no deaths. In total, three patients suffered from a documented post-operative complication.

Table A

	Pre-operative	Post-operative	p
Hb (g/dl)	12.1 (±0.3)	9.8 (±0.2)	<0.001
WCC ($x10^9$)	7.7 (±0.4)	10.4 (±0.5)	<0.001
C-Reactive Protein (mg/l)	2.1 (0.4-271.3)	133.4 (16.4-279.2)	<0.001
LOS (days)	-	11 (8-31)	-
Complications (N^o)	-	3	-

Table B

	Complications	p (Chi^2)
Gender		
Males	2 (67%)	0.765
Females	1 (33%)	
ASA Score		
I	1 (33%)	
II	1 (33%)	0.074
III	1 (33%)	
Dukes' Stage		
A	1 (33%)	
B	1 (33%)	0.918
C	1 (33%)	

Table 4.2 A - Peri-operative details, B - Complications breakdown

A – Hb, haemaglobin; WCC, white cell count; both given as mean (±s.e.m.); LOS given as median (range). **B** - Complication results given as number (percentage).

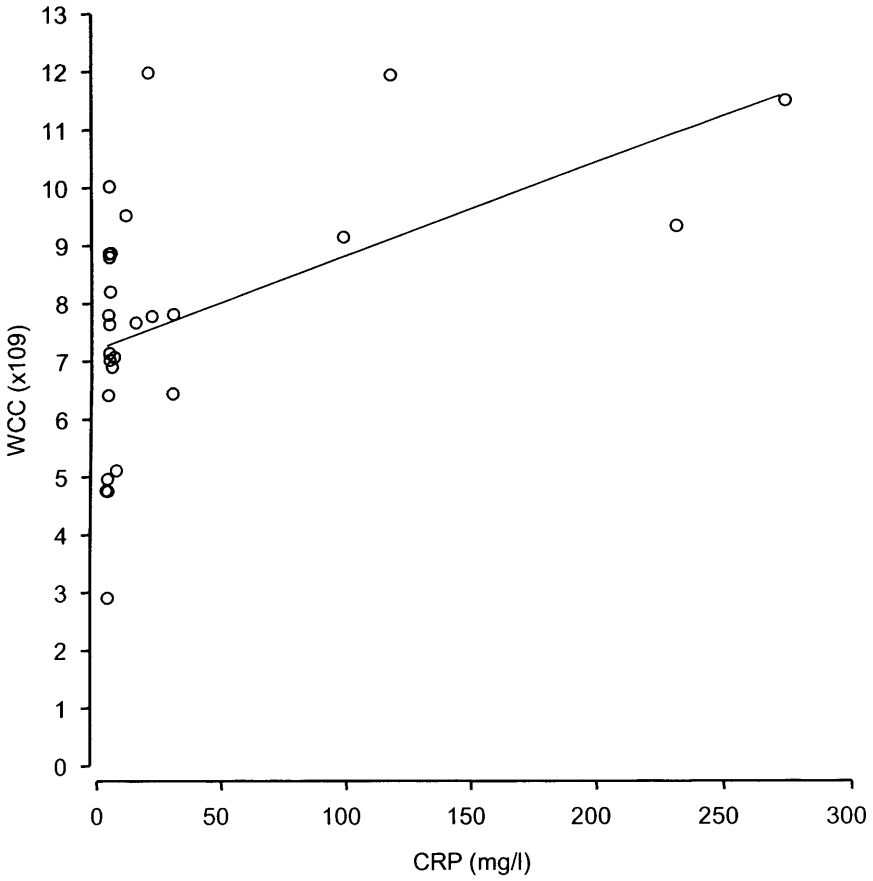


Figure 4.1 Pre-operative WCC and CRP

Dots represent individual patient results. There was a significant positive correlation between WCC and CRP pre-operatively ($p<0.001$, $R=0.593$, Spearman's rank correlation).

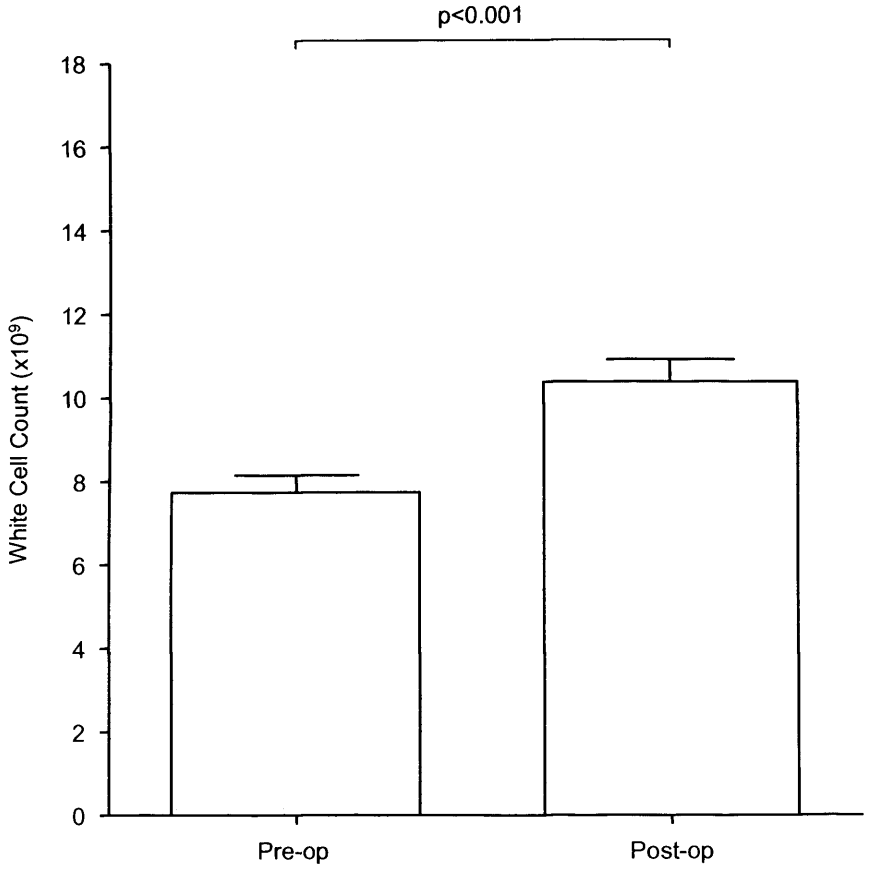


Figure 4.2 Peri-operative white cell count (WCC)

Bars represent mean values, errors are s.e.m. There was a significant rise in WCC following surgery ($p<0.001$, paired t-test).

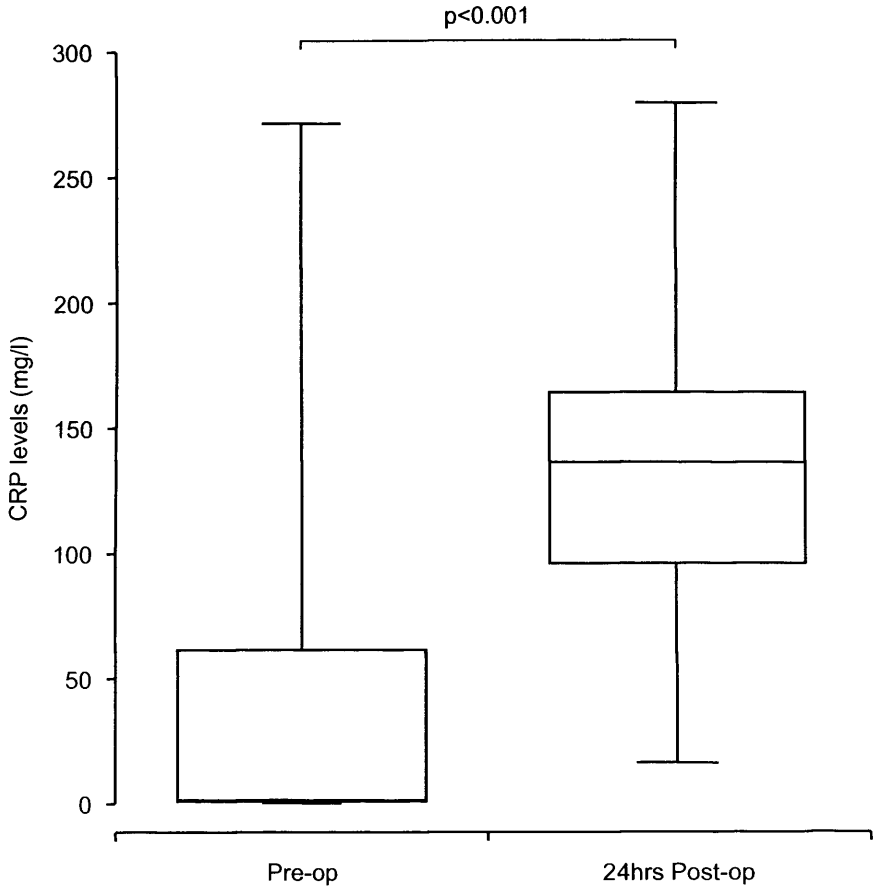


Figure 4.3 Peri-operative CRP

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a significant rise in CRP following surgery ($p<0.001$, Wilcoxon pairs).

A 47 year old man with no co-morbidity and an ASA I score, undergoing Abdomino-Perineal resection, developed a perineal wound infection, which subsequently dehisced. This was treated conservatively, with packing and antibiotic therapy, and the patient was able to be safely discharged on day 15, with further dressings in the community. A 77 year old woman, classed as ASA III due to her background of controlled hypertension and past cardiac failure, went into congestive cardiac failure following a sigmoid colectomy. She was stabilised with medical therapy, and discharged on day 21. Finally, an 87 year old man with no past medical history other than osteoarthritis, and undergoing a right hemicolectomy, developed an uncomplicated wound infection which responded well to antibiotics. He had a pre-operative ASA II score, and was discharged on post-operative day 11.

The development of complications, was not related to patient factors such as age ($p=0.590$, Mann-Whitney U, Table 4.3A), gender ($p=0.765$, Chi^2) or Dukes' stage ($p=0.918$, Chi^2; all Table 4.2B). Interestingly, the group who experienced a poorer outcome also did not differ in their operation time ($p=0.496$, Mann-Whitney U; Table 4.3A), which serves as a crude marker of severity of surgical experience. On the other hand, looking at a simple measure of the immune response to surgery, there was a greater percentage change in WCC in those developing complications (63.6 (±1.1) vs 36.6 (±8.3) %, complications *vs* no complications, $p<0.003$, unpaired t-test; Table 4.3A; Figure 4.4). On the other hand, no such association with the post-operative rise in CRP was observed ($p=0.314$, Mann-Whitney U; Table 4.3A).

Table A

	Complications	No Comps	p
Age	77 (47-87)	69 (43-81)	0.590
Pre-op Hb (g/dl)	11.1 (±0.9)	12.1 (±0.3)	0.381
Pre-op WCC ($x10^9$)	6.2 (±1.6)	7.9 (±0.4)	0.396
Pre-op CRP (mg/ml))	27.1 (1.0-129.7)	26.9 (0.4-279.2)	0.428
Operation time (mins)	235 (193-471)	227 (101-349)	0.496
% Change in Hb	29.8 (17-30)	18.9 (-19.8-39.7)	0.390
% Change in WCC	63.6 (±1.1)	36.6 (±8.3)	0.003
Change in CRP (mg/l)	94 (-11-129)	121 (14-252)	0.314

Table B

	LOS	p
Gender		
Males	13 (8-31)	0.53
Females	11 (8-25)	
ASA Score		
I	12.5 (10-15)	
II	11 (8-25)	0.216
III	18 (11-31)	
Dukes' Stage		
A	11 (9-21)	
B	11 (8-19)	0.334
C	15 (8-31)	

Table 4.3 A - Features of those with complications. B - LOS breakdown

Hb, haemaglobin; WCC, white cell count; both given as mean (±s.e.m.). LOS, length of stay, given as median (range). Non-parametric (Mann-Whitney U; Kruskal-Wallis) or Chi^2 test p-values given.

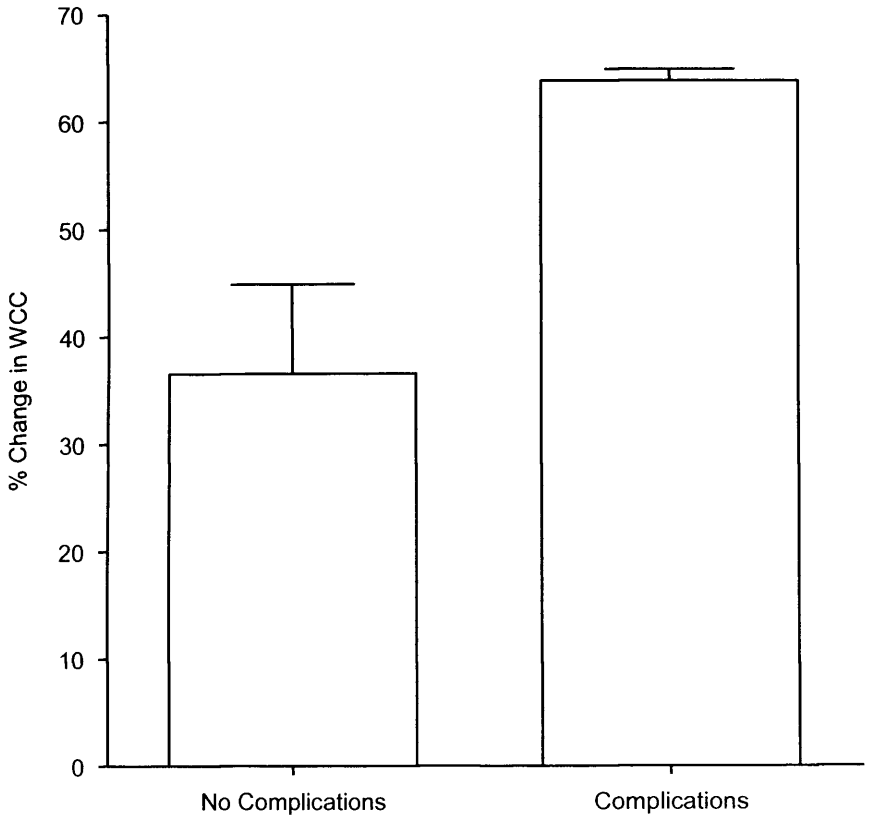


Figure 4.4 Comparing post-operative complications by percentage change in white cell count

Bars represent mean values, errors are s.e.m. Those who experienced post-operative complications had a greater WCC rise after surgery ($p<0.003$, unpaired t-test).

The overall post-operative length of stay (LOS) was median 11 (range, 8-31) days with no significant difference ($p=0.53$, Mann-Whitney U) between males (13 (8-31) days) and females (11 (8-25) days; Table 4.3B). Interestingly, again patient factors such as age ($p>0.6$, Spearman's Rank correlation), Dukes' stage ($p=0.334$, Kruskal-Wallis) and ASA score ($p=0.216$, Kruskal-Wallis) did not seem to affect LOS (Table 4.3B). However, as illustrated in Figure 4.5, operative procedure demonstrated a clear trend to affect LOS ($p=0.066$, Kruskal-Wallis). Conversely, there was no interrelation between operation time and LOS ($p>0.4$, Spearman's Rank correlation).

Given that several recent studies (Reddy *et al.,* 2003; Braga *et al.,* 2005) have found that 10 days is the median post-operative LOS following resection for colorectal cancer, an analysis of "short stay" (*ie* LOS=10 days) and "long stay" (*ie* LOS>10 days) may be of more relevance. However dividing the study population into these two groups and looking at potentially influential patient and operative characteristics, still did not reveal any particular differences; patient factors such as gender ($p=0.30$, Chi^2), age ($p=0.49$, Mann-Whitney U), ASA score ($p=0.32$, Chi^2) and pre-operative Hb ($p=0.22$), were not significantly different between the "short" and "long" stay patients (see Table 4.4). Similarly, operative factors such as percentage change in Hb ($p=0.72$) or operation time ($p=0.54$; all Mann-Whitney U) did not differ between these two groups either (Table 4.4). On the other hand, as with the patients suffering complications, there was a greater percentage change in WCC in the "long" stay patients ($p<0.05$, unpaired t-test; Table 4.4), as illustrated in Figure 4.6. However, there was no difference between these groups in terms of pre-operative ($p=0.26$) or post-operative change in CRP ($p=1.0$; both Mann-Whitney U; Table 4.4).

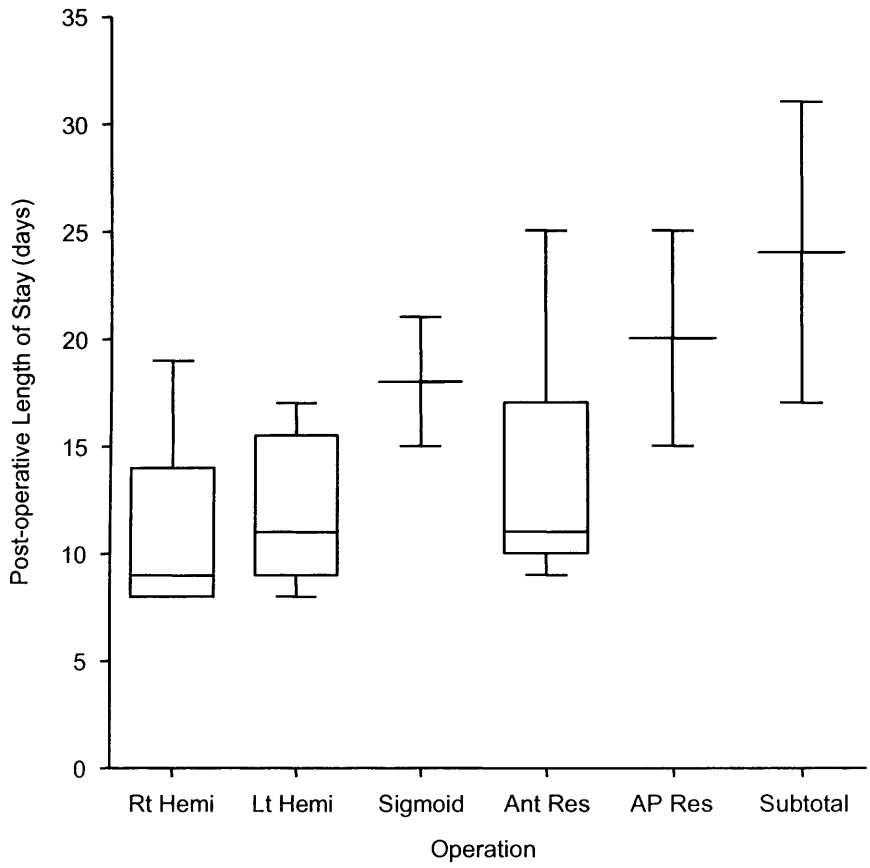


Figure 4.5 Post-operative length of stay by operation

Box and whisker plots representing median (line) values with quartiles (box) and range (error). A trend was observed when comparing LOS by operation undergone (p=0.066, Kruskal-Wallis).

	LOS =10 days	LOS >10 days	p
Number	9	20	-
Sex			
Male	4	13	0.30*
Females	5	7	
Age	69 (55-81)	70.5 (43-87)	0.49†
ASA Score			
I	1	1	
II	8	15	0.32*
III	0	4	
Pre-op Hb (g/dl)	11.5 (±0.6)	12.4 (±0.4)	0.22†
Pre-op WCC ($x10^9$)	8.0 (±0.5)	7.6 (±0.5)	0.60†
Pre-op CRP	101.1 (1.3-279.2)	17.8 (0.4-271.3)	0.26†
Operation time (mins)	203 (126-316)	231 (101-471)	0.54†
%Change in Hb	20.5 (-19.8-39.7)	18.7 (-10.1-38.8)	0.72†
% Change in WCC	21.2 (±7.8)	47.5 (±10.0)	<0.05‡
Absolute Change in CRP	105 (50-155)	121 (-11-252)	1.00†

Table 4.4 LOS breakdown variables

Values given as median (range) or mean (±s.e.m.). LOS, Length of stay. Hb. haemaglobin. WCC, white cell count. †Mann-Whitney U test. *Chi^2 test. ‡Unpaired t-test.

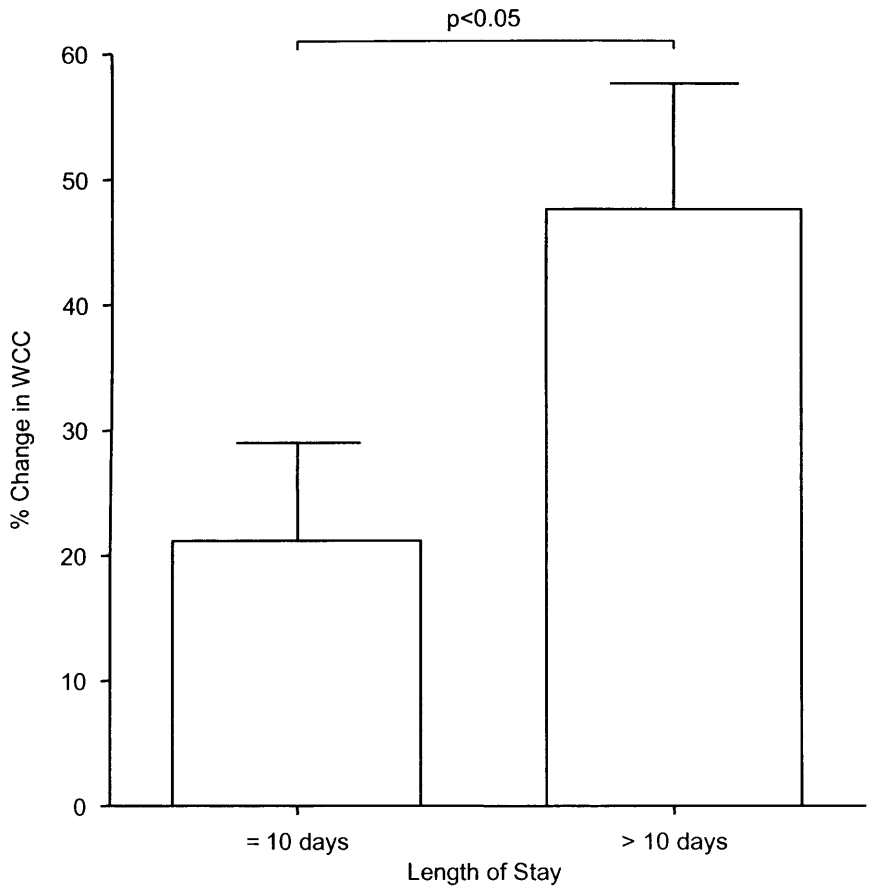


Figure 4.6 Comparing post-operative length of stay by percentage change in white cell count

Bars represent mean values, errors are s.e.m. Those with a post-operative stay >10 days had a significantly greater rise in WCC ($p<0.05$, unpaired t-test).

Patient genotypes

Summaries of results of patient genotyping and representative gel pictures are shown in Tables 4.5 and 4.6 and Figures 4.7 and 4.8. Both ACE I/D and IL6 -174 G/C polymorphisms were in Hardy-Weinberg equilibrium, with no significant differences between observed and expected genotype frequencies (ACE, p=0.39; IL6 p=0.96; Chi^2; Table 4.6).

Given that the percentage change in WCC following surgery was the only factor that differed significantly between the "long" and "short" stay groups of patients, it is reasonable to assess the genotypic influence over this. Starting with ACE, there seemed to be a difference in magnitude of WCC change, depending on genotype (23.1 (±5.7) *vs* 54.7 (±11.1) *vs* 22.7 (±17.1) %, I/I *vs* I/D *vs* D/D, p=0.109, ANOVA), with I/D heterozygotes showing a tendency towards a greater rise, as illustrated in Figure 4.9. However, no similar such relationship was present with the IL6 genotype (p=0.9, ANOVA).

An analysis of the relationship between genotype and outcome is made later in Chapter 7, to allow combination with results from the gene expression assay experiments; this may provide evidence for whether any observed polymorphic association with outcome is due to a functional genetic effect.

	Male	Female	Total
ACE Genotype			
I/I	3 (43%)	4 (57%)	7 (24%)
I/D	11 (73%)	4 (27%)	15 (52%)
D/D	3 (43%)	4 (57%)	7 (24%)
IL6 Genotype			
G/G	8 (53%)	7 (47%)	15 (52%)
G/C	6 (60%)	4 (40%)	10 (34%)
C/C	3 (75%)	1 (25%)	4 (14%)

Table 4.5 ACE and IL6 Genotype frequencies

Percentage values given in parentheses.

Locus	Allele	Frequency	n	Genotype	Observed Frequency	Expected Frequency	?^{2}	p
IL6 -174	G	0.69	20	GG	0.52	0.48		
				GC	0.34	0.43	1.87	0.39
	C	0.31	9	CC	0.14	0.10		
ACE	I	0.50	15	II	0.24	0.25		
				ID	0.52	0.50	0.08	0.96
	D	0.50	15	DD	0.24	0.25		

Table 4.6 ACE and IL6 Genotype allele breakdown

No significant differences between the observed genotype frequencies and those predicted by the Hardy-Weinberg equilibrium (Chi^2 test).

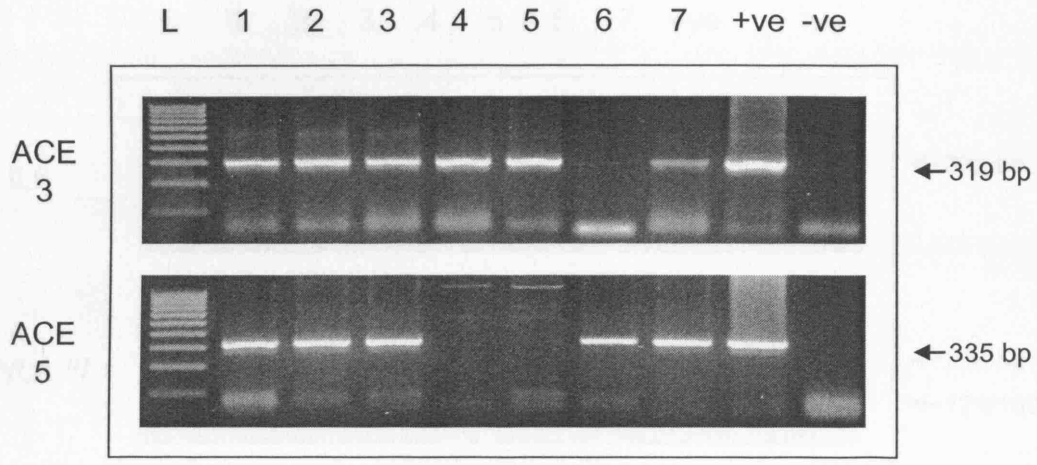


Figure 4.7 ACE genotype determined by PCR

Representative picture of ACE 3 and ACE 5 primer PCR products. L, 100bp molecular standard ladder; 1-7, patient samples; +ve, positive control (I/D heterozygote); -ve, negative control. Two primer sets used, due to preferential amplification of D allele with ACE 3 primers.

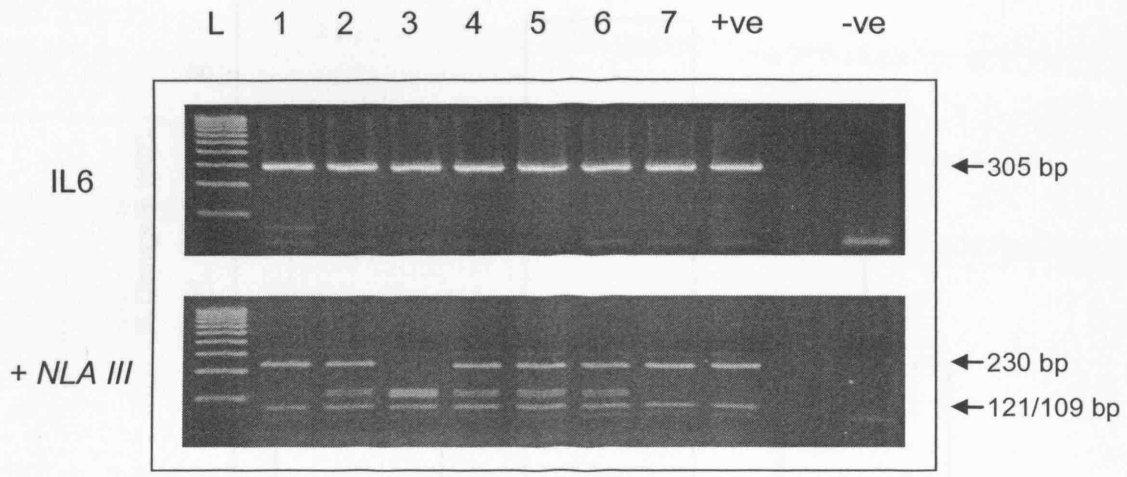


Figure 4.8 IL6 -174 G/C genotype determined by PCR and *NLA III* restriction

Representative picture of IL6 genotype primer PCR products and *NLA III* restriction products. L, 100bp molecular standard ladder; 1-7, patient samples; +ve, positive control (I/D heterozygote); -ve, negative control. 1, G/G; 2, G/C; 3, C/C.

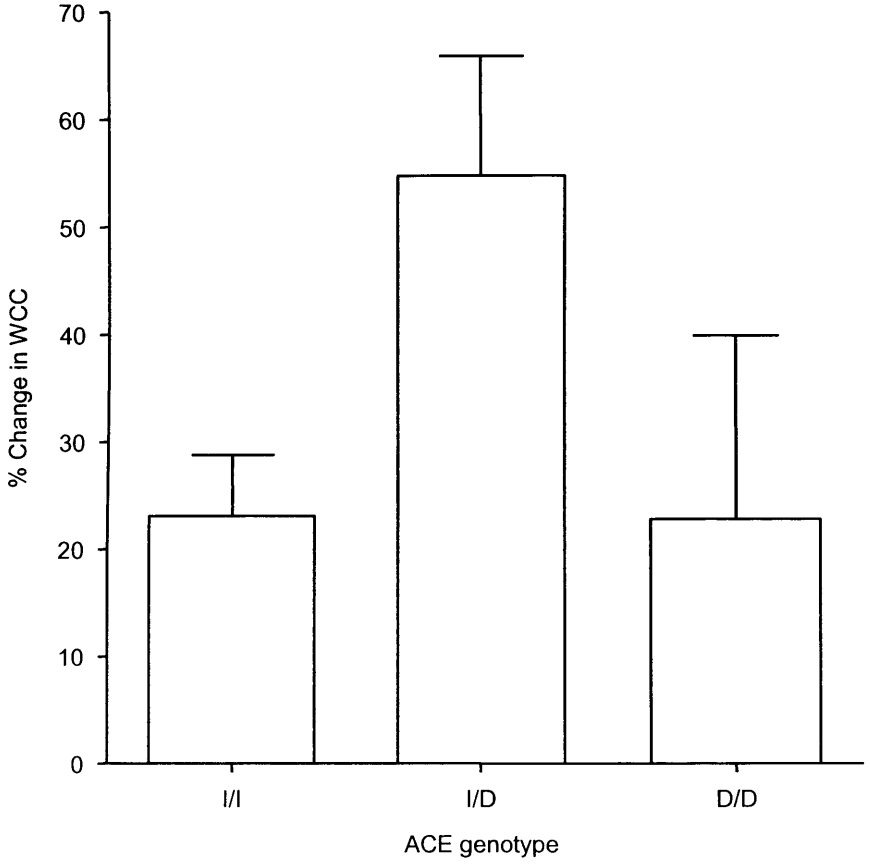


Figure 4.9 Percentage change in WCC by ACE genotype

Bars represent mean values, errors are s.e.m. Although not reaching statistical significance, there was some evidence of a difference between genotypes (p=0.109, ANOVA).

4.4 DISCUSSION

Colorectal cancer is rare before the age of fifty, and its incidence is strongly related to age, with around 85% of cases arising in the over 60 year old age group (Cancer Research, 2006). Tumours are more likely to affect the left colon than the right, with the sigmoid colon, recto-sigmoid region and colon together accounting for over half the cases (Cancer registration statistics, 2005). Although not the case in younger patients, it is more common in men, with sex ratios (male/female) of 1.4 to 1.7 being documented; an effect that is more pronounced in cases affecting the left colon and rectum (Faivre *et al.,* 2002). Thus, the age of patients, slight male sex preponderance and distribution of disease site, with consequent operative procedure undergone, of this study cohort (see Table 4.1) were consistent with other reports (Fazio *et al.,* 2004).

The finding that ASA II was the commonest assessment score (see Table 4.1) for pre-operative fitness in this sample population is unsurprising given the median age of 69 years, and corresponds to findings from larger series of colorectal cancer patients (Fazio *et al.,* 2004). Likewise, the even distribution of Dukes' stage within the study population (see Table 4.1) makes it more likely that any observed effect was due to the trauma of surgery, rather than the underlying cancer. Thus in summary, this study population was a typical group of patients, with no evidence of selection bias, in terms of age, sex, ASA, Dukes' stage or tumour location, when compared to previously published reports.

The grouping of patients undergoing both colon and rectum resections together, to produce a study population has been previously employed when investigating the peri-operative cytokine response (Miki *et al.,* 2005). Importantly, the fact that all the

patients were Caucasian is relevant, as past studies have shown a racial difference in the relationship between ACE genotype and activity (Bloem *et al.,* 1996).

Moving on to the immune and inflammatory reaction to surgery, the significant rise in C-reactive protein (CRP) observed at 24hrs following surgery reflects the expected acute phase response (see Figure 4.3). This is in line with other studies, where it has proved to be a consistent and reliable marker of surgical stress (Halevy *et al.,* 1995; Hill *et al.,* 1995). Some have sought to use CRP as a potential prognostic factor, for post-operative complications and overall LOS; serial measurement has been predictive of septic complications following abdominal surgery (Mustard *et al.,* 1987) and a one-off pre-operative assessment has been associated with both complications and length of stay post-cardiac surgery (Fransen *et al.,* 1999). However, in this study, no such association between CRP and outcome was observed, which may have been due to the relatively small cohort size or the choice of a one-off 24 hour post-operation sampling timepoint. Indeed, it may be that the rate of increase, or the peak CRP result may have been of greater relevance.

WCC is a simple indicator of systemic inflammation and stress in the ill patient. It is routinely measured during clinical practice in the assessment of potential infection, but like CRP, it lacks specificity. Furthermore, the extent of WCC change has been used as a marker to compare the immune response to laparoscopic and open surgery (Rahr *et al.,* 2006). In addition, measurement of the peri-operative WCC response to distinguish between the systemic inflammatory response syndrome (in which no documented infection is present) and sepsis has been documented (Mustard *et al.,* 1987). However, there is no evidence in the literature of using a peri-operative WCC change as a post-

operative prognostic indicator of outcome. In this study, a significant post-operative rise in WCC was observed (see Table 4.2A and Figure 4.2), which corresponds to previous findings (Sarbinowski *et al.,* 2005; Kim *et al.,* 2006). Such a leucocytosis is in keeping with the well established acute inflammatory response that is known to be provoked by the surgical stimulus (as discussed in Section 1.1).

There were no in-hospital deaths following surgery in the study group of patients, which although excellent, is unsurprising given the relatively small cohort size and low expected mortality rate for elective colorectal surgery (5.7%; Longo *et al.,* 2000). Mortality was thus an unsuitable choice of outcome measure. Similarly, there were only 3 patients with post-operative complications (see Table 4.2B), making it difficult to use this as the sole outcome measure for analysis. Thus, overall post-operative length of stay (LOS) was also employed as a marker of outcome. Its use can be justified medically, with past studies demonstrating that post-operative complications, such as cardiopulmonary problems and wound infection, are the biggest factors underlying prolonged stay (Tartter, 1996; Lawrence *et al.,* 1995). In addition, and of increasing relevance in the modern NHS with its resource limitation, is the economical argument. The cost of a day's stay on a surgical ward has been estimated at £220 and rising (Schwartz *et al.,* 1991; Braga *et al.,* 2005), making LOS a particularly fond outcome measure for managers. In fact, LOS has even been suggested as a prognostic indicator following colorectal surgery, with those having a shorter stay likely to have a longer disease-free survival period (Tartter, 1996).

In any case, an obvious confounding factor for the use of LOS is "social" delay, when a patient is medically fit for discharge, but unable to go home due to inadequate social

circumstances. However, with the prospective gathering of data, there was no doubt that no such delays to discharge occurred within this particular study group. Similarly, all the patients were operated on within the same institution, and under the care of only one colorectal surgeon. Thus, protocols for pre-, peri- and post-operative management of patients were standardized for the whole study group. This is of importance, as an individual surgeon's management choices will clearly affect the patient's LOS. For example, decisions regarding per-operative drain placement, subsequent removal, and the pace of post-operative diet advancement, have all been found to affect LOS (Binderow *et al.,* 1994). In addition, all the patients were subject to the same standard discharge criteria of restored bowel function, adequate mobility and oral pain relief, and normal oral intake, further eliminating surgeon or institution policy as another possible LOS confounding factor.

No difference in outcome, as determined by LOS, was found between the sexes, or when grouping patients by ASA score (Table 4.3B). The latter may seem unusual, but is in agreement with a recent UK study of 350 patients undergoing colorectal cancer surgery (Reddy *et al.,* 2003). On the other hand, a much larger US study of over 5000 patients did find a poorer outcome with ASA III (Longo *et al.,* 2000). The only factor that had any bearing on outcome in this study was the type of procedure undergone, with patients having a right hemicolectomy having a relatively shorter LOS (Figure 4.5); again in agreement with previous series (Tartter, 1996). However, it is probably not sensible to focus on the type of operation undergone, given the wide spectrum of complexity that may be encountered when carrying out a particular procedure. Instead, the length of time spent in theatre has previously been chosen as probably representing a better indicator of operative difficulty and severity of surgical insult (Reddy *et al.,*

2003). Consequently, in this study no correlation between operation time and LOS was evident, which is consistent with other published work (Reddy *et al.*, 2003).

The relatively small number of patients successfully recruited to the study population is an obvious problem when trying to draw significant conclusions. Hence the decision to divide the patients into "short" (=10 days) and "long" (>10 days) stayers, with the dividing line being the average LOS from other published colorectal surgery series (Reddy *et al.*, 2003). Such an approach has been used previously in the analysis of post-operative colorectal LOS (Tartter, 1996) although a more up to date cut off figure (Reddy *et al.*, 2003) for spliting the study cohort into two groups was employed here. In fact, despite such a grouping strategy, there was still no difference in patient factors, such as age, ASA score, Dukes' stage, pre-operative Hb and pre-operative WCC between the "short" and "long" stayers (Table 4.4). Similarly, surgical factors such as operation time and change in Hb did not differ between these two groups either (Table 4.4).

The only significant finding was that the "short" stayers had a smaller percentage rise in WCC at 24 hours following surgery compared to those with a prolonged stay (Table 4.4; Figure 4.6). The relevance of WCC has been discussed earlier, and this result would seem to be a novel one. A possible explanation for the advantageous effect of a limited WCC rise may be the avoidance of an exaggerated response to injury. Such a hyper-inflammatory state may lead to the development of a systemic inflammatory response syndrome (SIRS), which may even paradoxically result in immunosuppression (Davies *et al.*, 1997). Either of these can increase the risk of post-operative morbidity and ultimately prolong LOS (Novitsky *et al.*, 2004). Interestingly, although there were

only three patients who experienced post-operative complications, the post-operative percentage rise in WCC was also greater in this group than those who had an uncomplicated recovery (Table 4.3A; Figure 4.4). As has been discussed earlier, it is difficult to extrapolate this finding due to the small nature of this study, but it would certainly warrant further investigation with larger numbers.

Both ACE and IL6 genotypes were in Hardy-Weinberg equilibrium, and there was no relationship with Dukes' stage (see Table 4.6). Although much work has concentrated on the genetic basis of colorectal cancer pathogenesis, neither ACE nor IL6 have been implicated, making it unsurprising that there was no preponderance of any particular genotype within the study cohort.

Given that the percentage change in WCC seemed to be of potential importance, the genotypic influence over this was examined. Despite there being no clear difference in WCC response between patients with different ACE genotypes, there was a definite tendency for I/D heterozygotes to produce a greater elevation (Figure 4.9). Past studies have examined the potential influence of ACE alleles by grouping subjects according to whether or not they possess a particular allele (Montgomery *et al.,* 1999; Woods et al 2001). This obviously has the advantage of dividing the study population into only two groups, which is particularly attractive with a small cohort, such as in this case. However, it does assume that the alleles do not behave in a co-dominant or "additive" fashion. The application of such an approach to this data would not be of value, as the heterozygote subjects were responsible for the majority of the observed rise in WCC. This is illustrated in Figure 4.10, demonstrating that a greater rise in WCC can be attributed to possession of either I- or D-allele, depending on the choice of grouping.

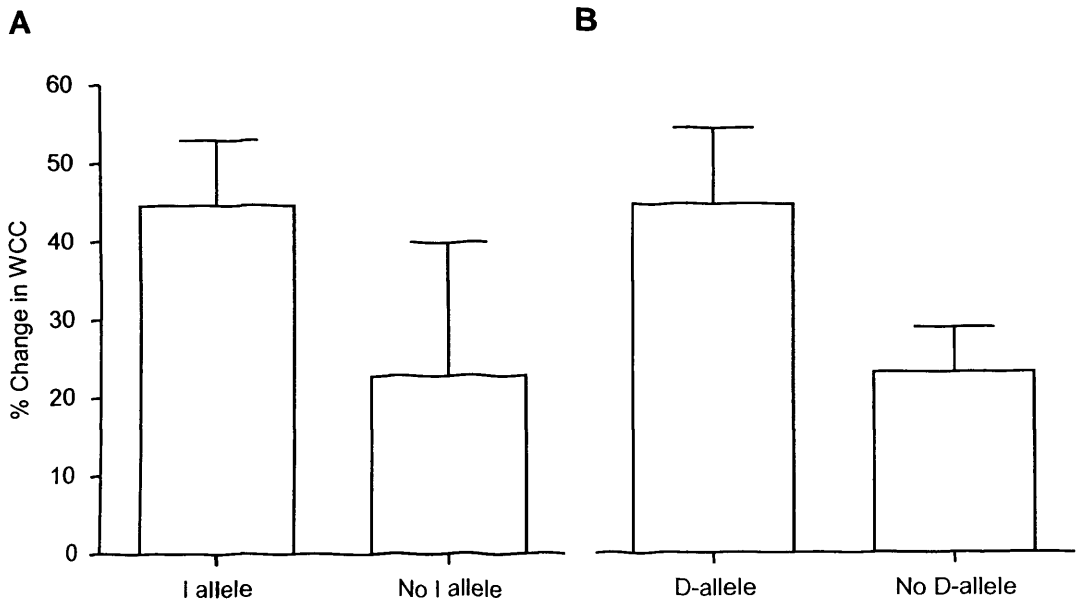


Figure 4.10 Percentage change in WCC by ACE allele

Bars represent mean values, errors are s.e.m. Patients grouped according to possession of I-allele (**A**) or D-allele (**B**). The dominant influence of I/D heterozygotes makes grouping according to possession of either allele futile.

A summary of the main findings from this investigation of colorectal cancer patients undergoing elective surgery are given below:

- There was no pre-ponderance of any particular ACE I/D or IL6 -174 G/C genotype within the study population (Table 4.5).
- Surgery elicited significant rises in both CRP and WCC (Figures 4.2 and 4.3).
- There was no association between age, gender, ASA score or operation length and LOS, even when grouping the study population into patients with LOS = 10 days and the remainder with a longer stay (Tables 4.3 and 4.4).
- Patients developing complications had a greater percentage change in WCC at 24 hours following surgery compared to those with an uncomplicated recovery (Figure 4.4).
- Short-stay patients had a smaller percentage rise in WCC at 24 hours following surgery compared to those with a prolonged stay (Figure 4.6).
- Despite there being no clear evidence of association between ACE or IL6 genotypes and the WCC response to surgery, a definite tendency for ACE I/D heterozygotes to produce a greater elevation was observed (Figure 4.9).

CHAPTER 5

Surgery, IL6 -174 G/C Genotype and Expression

CHAPTER 5

5.1 INTRODUCTION

IL6 is a pleotropic cytokine that acts as a highly inducible essential mediator of the acute phase response. It is constitutively expressed in peripheral blood leucocytes, spleen and liver, and has been found to be inducible in nearly every human tissue and cell type (Biffl *et al.,* 1996). By stimulating the production of further pro-inflammatory mediators such as CRP and a2-macroglobulin, IL6 modulates the local and systemic inflammatory responses (Gauldie *et al.,* 1987; Castell *et al.,* 1989).

The serum IL6 response to surgery has been related to the extent of surgical trauma (Hildebrandt *et al.,* 2003), and its duration has been found to last up to 72hrs (Baigrie *et al.,* 1992). A number of different cohorts have been subject to such investigation, including AAA patients (Bown *et al.,* 2004), CABG patients (Galley *et al.,* 2003) and colorectal cancer patients (Miki *et al.,* 2005) (see Section 1.1). These studies strived to identify a prognostic role for the peri-operative IL6 response, with varying degrees of success. However, the role of the functional -174 G/C polymorphism in determining the IL6 response to surgery has received less attention and yielded mixed results. Both CC homozygotes (Woods *et al.,* 2001) and GG homozygotes (Gaudino *et al.,* 2003) have been associated with a greater response to CABG surgery. Importantly, these studies only looked at IL6 protein levels, without addressing the influence of surgery on gene transcription. Given the short half life, it is thought that circulating levels of IL6 are mostly regulated at the level of transcription (Castell *et al.,* 1988). It is thus reasonable to study both the protein and gene transcription responses to colorectal cancer surgery, and the role of the -174 G/C polymorphism in determining this reaction.

5.2 MATERIALS AND METHODS

As described in Chapter 3, following ethical approval and informed consent, blood samples were taken from elective colorectal cancer surgery patients pre-operatively and, when possible, 4 hours, 24 hours, 10 weeks and 20 weeks following surgery. PBMNs and plasma were separated from blood by a process of centrifugation through a density gradient polymer and from the former, nucleic acids were extracted using a phenol-ethanol precipitation method (see Sections 3.2 and 3.3). An RT reaction was performed on the extracted RNA to produce cDNA (see Section 3.6). Primers specific for the IL6 gene were designed, and a PCR used to amplify mRNA transcripts, so that a semi-quantitative method of measuring gene transcription could be used. These PCR products were separated by agarose gel electrophoresis, and analysed by scanning densitometry. Results were normalised was by comparing transcription to that of the standard house-keeping gene GAPDH-3 (see Section 3.6 for the full protocol). Each sample was run twice, and the final results correspond to the mean of these two experiments.

Levels of IL6 protein were assessed using an ELISA (see Section 3.8), with samples run in duplicate, and the final result representing the mean of two separate experimental plates. An estimation of experimental accuracy was made by evaluating the inter- and intra-experimental variation; in this case, for IL6, the co-efficient of variation was 14% for intra-assay determinations, and 17% for inter-assay determinations. However, when discounting those assays that produced undetectable levels of IL6, these markers of experimental consistency improved to 9% for intra-assay and 11% for inter-assay variations.

5.3 RESULTS

Gene transcription

The study population consisted of the same cohort of patients described in Chapter 4. A representative photo of an agarose gel with separated RT-PCR products analysed semi-quantitatively for IL6 gene transcription is shown in Figure 5.1. The raw IOD results are included in Appendix IV. These values failed the Kolmogorov-Smirnov test for normality, and subsequently, non-parametric statistical tests were employed when making group comparisons.

At the pre-operative baseline timepoint, there was no difference in levels of IL6 gene transcription when comparing by ASA score (p=0.447, Kruskal-Wallis) or Dukes' Stage (p=0.521, Kruskal-Wallis). However, as seen in Figure 5.2, there was seemingly a trend towards elevated baseline transcription in females (0.159 (0.069-0.357) *vs* 0.118 (0.033-0.278) IOD, median (range); p=0.114, Mann-Whitney U). There was no evidence of a relationship between IL6 transcription and G/C genotype (p=0.965, Kruskal-Wallis, Figure 5.3). In fact, even grouping patients according to the presence or absence of a C allele failed to reveal any significant differences (p=0.593, Mann-Whitney U).

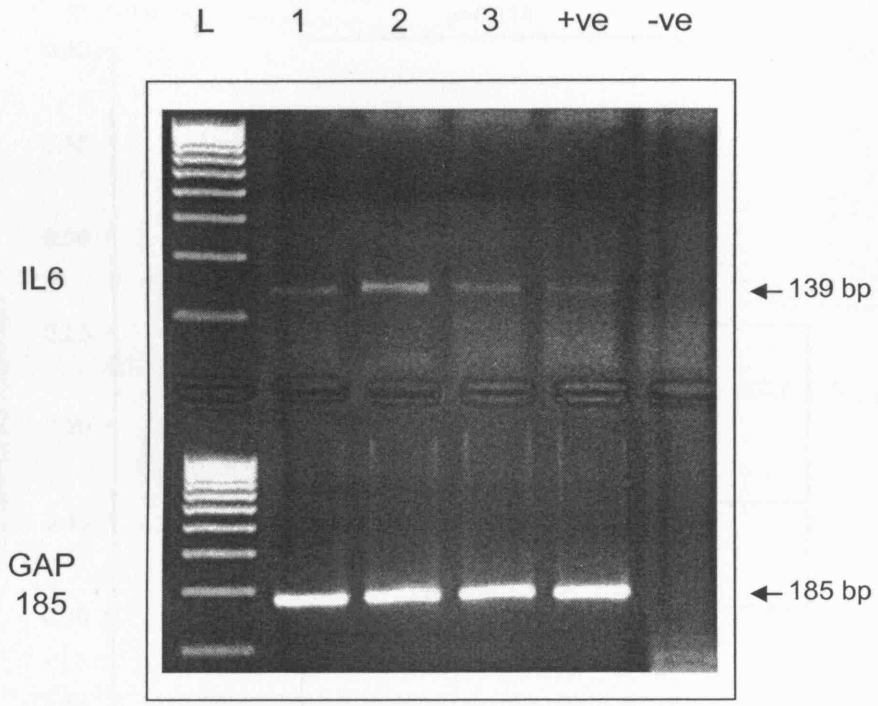


Figure 5.1 IL6 gene transcription determined by RT-PCR

Representative picture of IL6 PCR products. L, 100bp molecular reference ladder; 1-3, patient samples; +ve, positive control; -ve, negative control. GAPDH-3 used as internal control for the PCR reaction; respective gene transcript sizes (bp) are indicated.

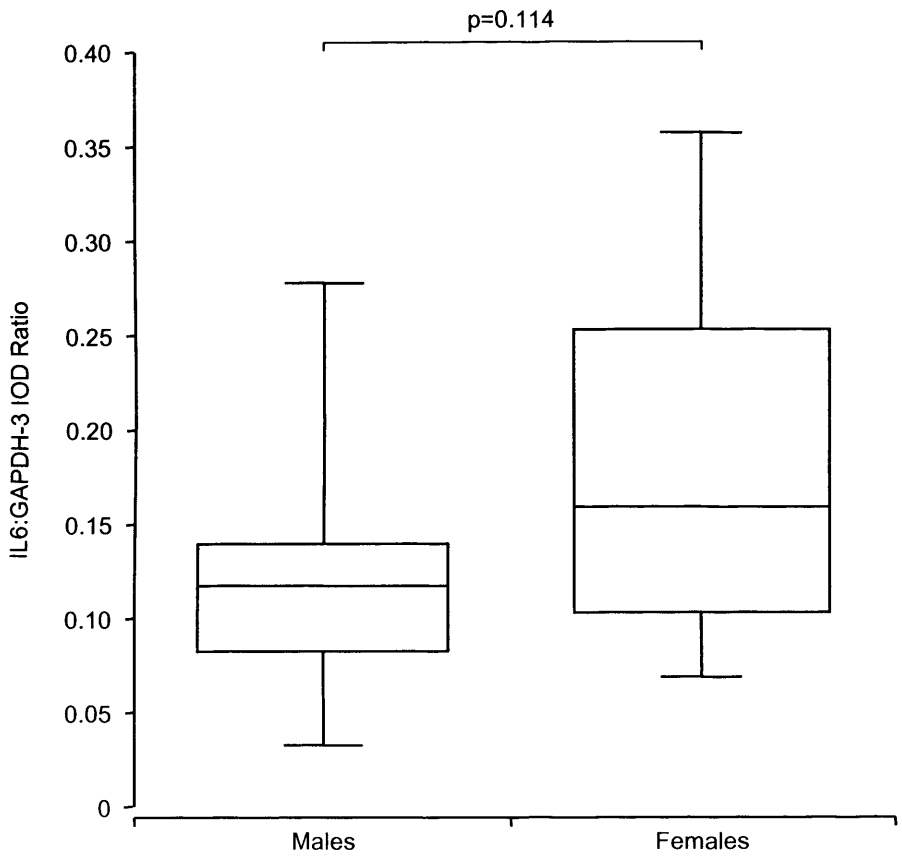


Figure 5.2 Pre-operative IL6 gene transcription by gender

Box and whisker plots representing median (line) values with quartiles (box) and range (error). IOD, total integrated optical density of IL6:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. There was evidence of a trend towards greater baseline pre-operative IL6 transcription in women (p=0.114, Mann-Whitney U).

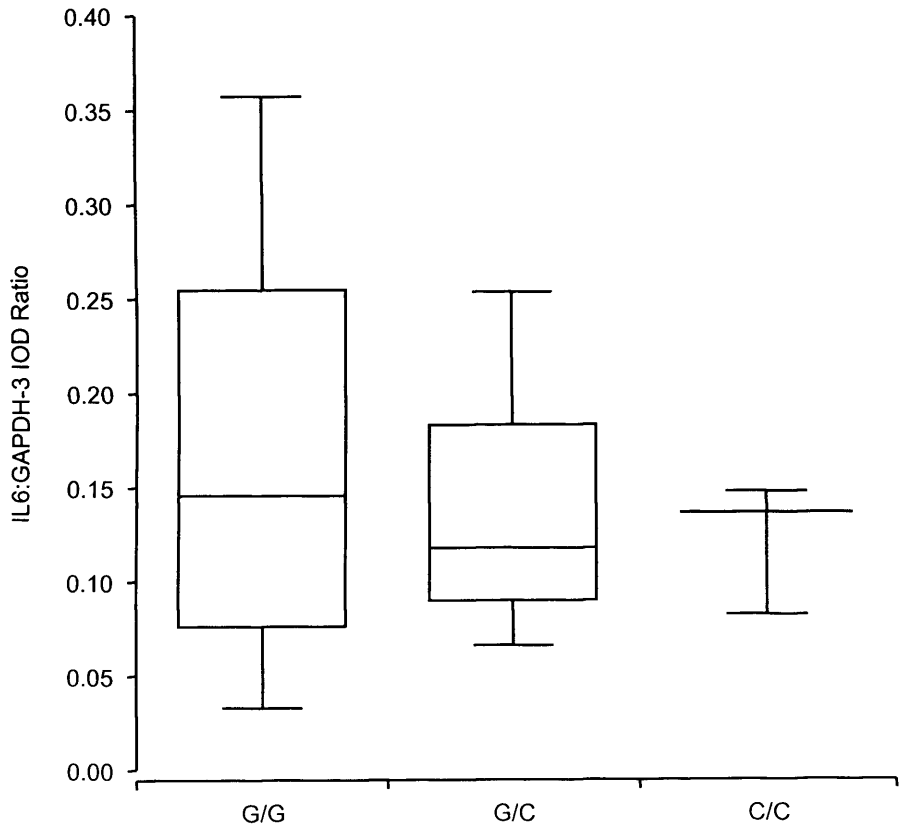


Figure 5.3 Pre-operative IL6 gene transcription by G/C genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). IOD, total integrated optical density of IL6:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. There was no evidence of any genotypic influence on transcription (p=0.965, Kruskal-Wallis).

Levels of IL6 gene transcription, at the discrete timepoint 24 hours following surgery, were again not influenced by Duke's stage (p=0.777, Kruskal-Wallis) or ASA (p=0.377, Kruskal-Wallis). Interestingly, any semblance of a sex-related trend towards greater transcription in females was no longer present 24 hours following surgery (p=0.426, Mann-Whitney U). However, post-operatively, the G/C genotype did seem to have a degree of influence on transcription levels, as seen in Figure 5.4; despite initial analysis not demonstrating any real difference between the 3 genotypes (p=0.159, Kruskal-Wallis), subgroup analysis revealed trends towards the C/C homozygotes (0.076 (0.053-0.098) IOD) having lower transcription than both C/G heterozygotes (0.229 (0.074-0.348) IOD; p=0.068, Mann-Whitney U) and G/G homozygotes (0.198 (0.062-0.699) IOD; p=0.076, Mann-Whitney U). In fact, if patients were grouped according to the presence (G/G and G/C) or absence (C/C) of a G-allele, then this trend to lower transcription levels in the latter remained (0.200 (0.062-0.699) *vs* 0.076 (0.053-0.098); p=0.055, Mann-Whitney U).

Moving on to the peri-operative response to surgery, graphically there seemed to be a rise in levels of IL6 gene transcription at 4hrs and 24hrs post-procedure, although this was not borne out by statistical analysis (p=0.237, Kruskal-Wallis, Figure 5.5). On closer analysis, at 24 hrs there was evidence of a rise in the median level of transcription (0.124 (0.033-0.357) *vs* 0.198 (0.053-0.699) IOD (median (range)); p=0.077, Mann-Whitney U, Figure 5.6), which was not evident at the earlier 4 hrs sampling point (0.120 (0.067-0.843) IOD; p=0.889, Mann-Whitney U). Concentrating on the scale of this response, as measured by percentage change in gene transcription, there was no evidence that the G/C genotype exerted any influence (p=0.831, Kruskal-Wallis, Figure 5.7).

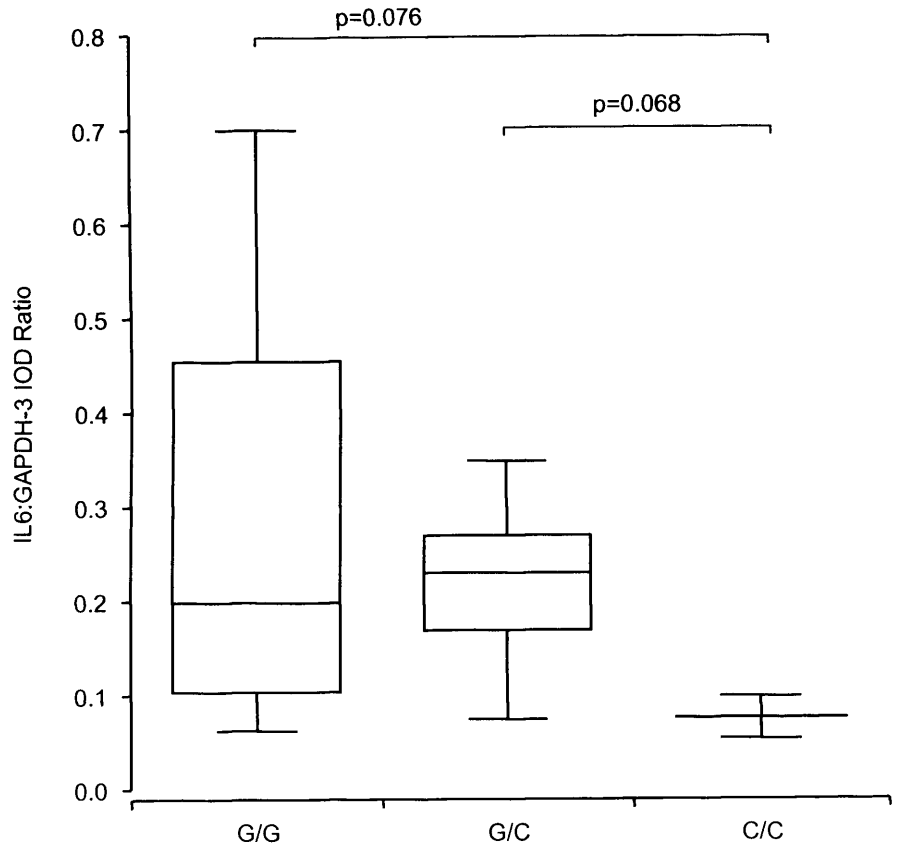


Figure 5.4 Post-operative IL6 gene transcription by G/C genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). IOD, total integrated optical density of IL6:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. Despite no initial evidence of a genotypic influence on transcription (p=0.159, Kruskal-Wallis), sub-group analysis did reveal differences between C/C and G/C (p=0.068) and G/G (p=0.076; both Mann-Whitney U) patients.

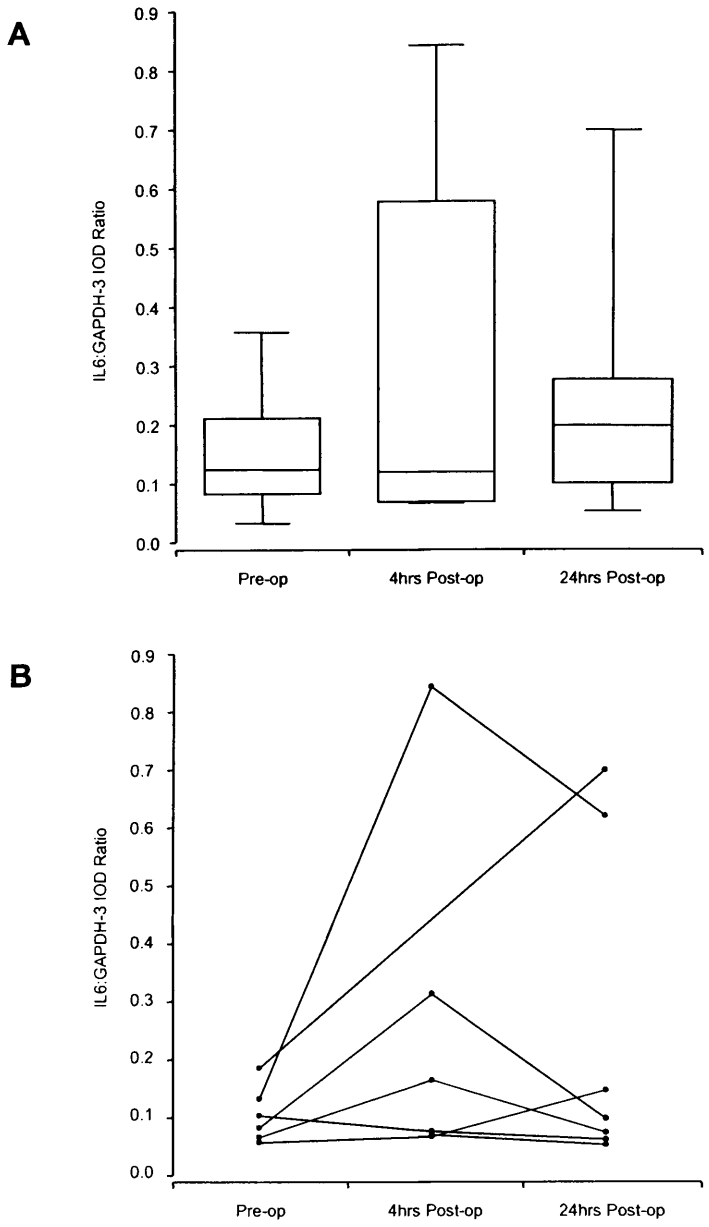


Figure 5.5 Peri-operative IL6 transcription

A, Box and whisker plots representing median (line) values with quartiles (box) and range (error). There appeared to be no difference between the groups (p=0.237, Kruskal-Wallis). **B**, Line plot representing results for individual patients with samples available at all 3 peri-operative time points. IOD, total integrated optical density of IL6:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses.

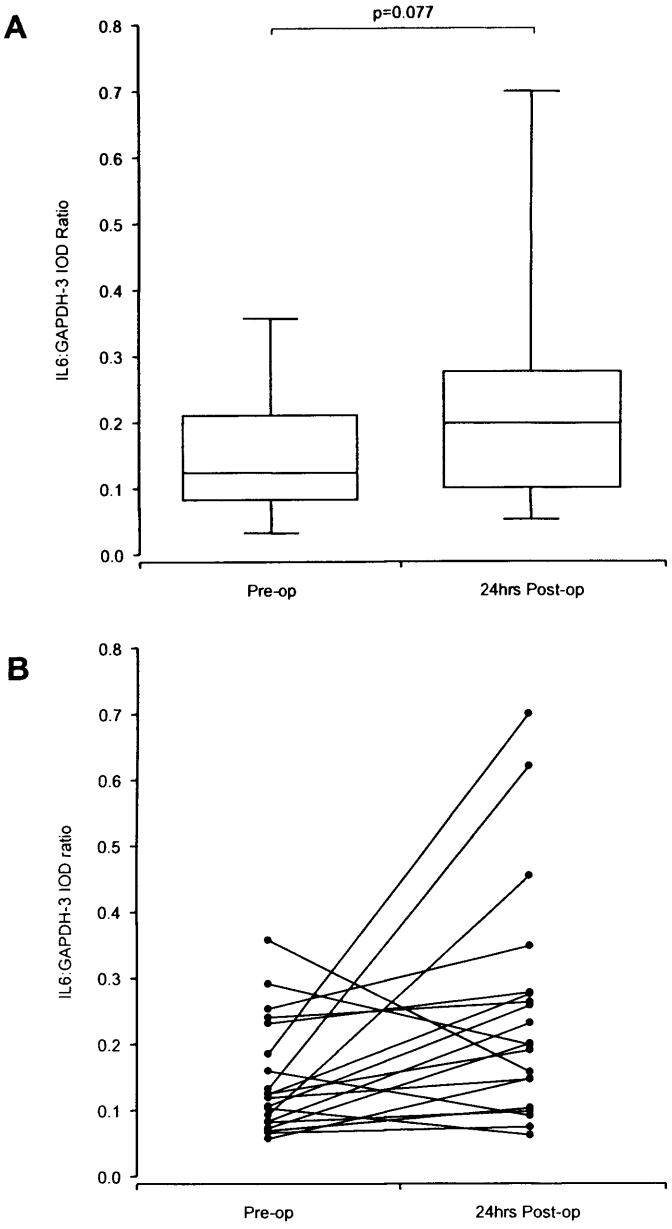


Figure 5.6 Post-operative change in IL6 transcription

A, Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a trend towards greater transcription at 24 hours (p=0.077, Mann-Whitney U). **B**, Line plot representing results for individual patients with samples available at both pre-operative and 24 hours post-operative time points. IOD, total integrated optical density of IL6:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses.

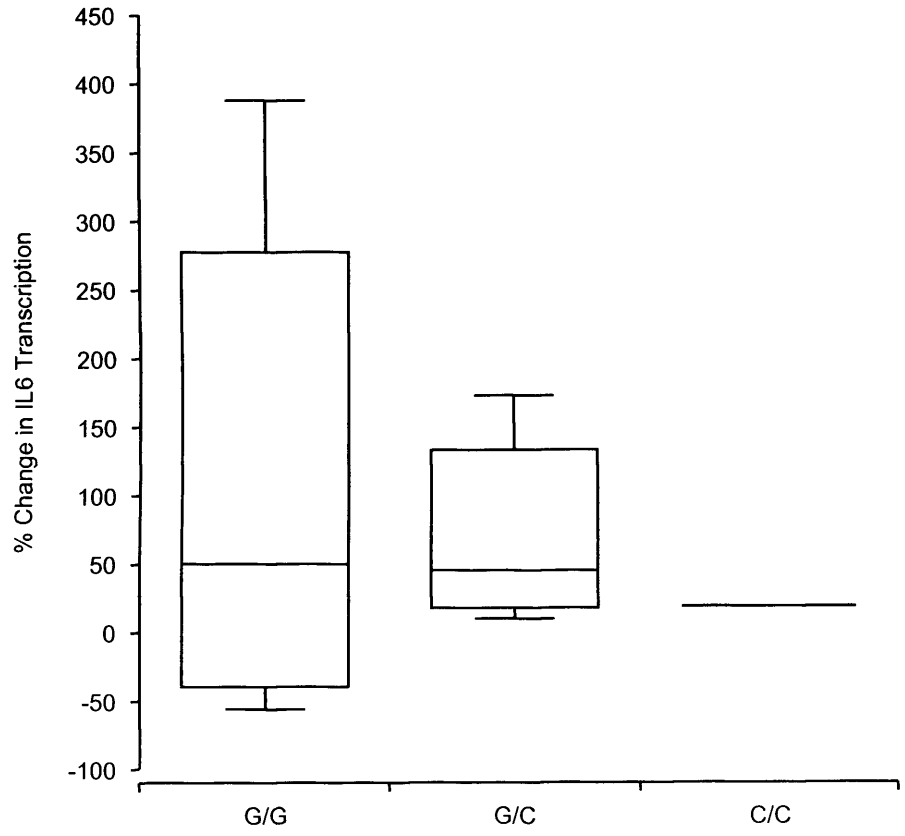


Figure 5.7 Post-operative change in IL6 transcription by G/C genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was no significant difference between genotypes in change in IL6 transcription following surgery (p=0.831, Kruskal-Wallis).

Even taking into consideration the small number of C/C homozygotes within the sample population, by grouping them with the G/C heterozygotes, thereby allowing a comparison between those who do and don't possess a C-allele, failed to demonstrate any significant differences (p=0.593, Mann-Whitney U). However, interestingly, the male patients launched a significantly greater transcription response to surgery compared to the women (133 (13-388) *vs* -11 (-56-172) %; p=0.014, Mann-Whitney U, Figure 5.8). On the other hand, there was no correlation between operation length, which acts as a surrogate marker for the extent of injury, and the change in IL6 transcription (p=0.431, Spearman's rank correlation). Indeed, there was no difference in response, when compared by operation undergone (p=0.170, Kruskal-Wallis). There was no other patient or operative factor that seemed to exert any influence on the transcription response.

Plasma protein expression

Plasma protein results (see Appendix V) were also found not to be distributed normally when evaluated with the Kolmogorov-Smirnov test, thus again prompting the use of non-parametric analyses.

The pre-operative levels of IL6 were undetectable in the majority (79%) of cases, making any further analysis of this baseline timepoint impossible. However, there was an overwhelming rise in IL6 concentration following surgery, peaking at 4hrs and remaining elevated at 24hrs (p<0.001, Kruskal-Wallis, Figure 5.9); closer analysis demonstrated the group medians to have risen significantly from baseline pre-operative levels (0 (0-32) ng/ml *vs* 157 (28-480) ng/ml at 4hrs, p<0.001, Mann-Whitney U; *vs* 112 (5-321) ng/ml at 24hrs, p<0.001, Mann-Whitney U).

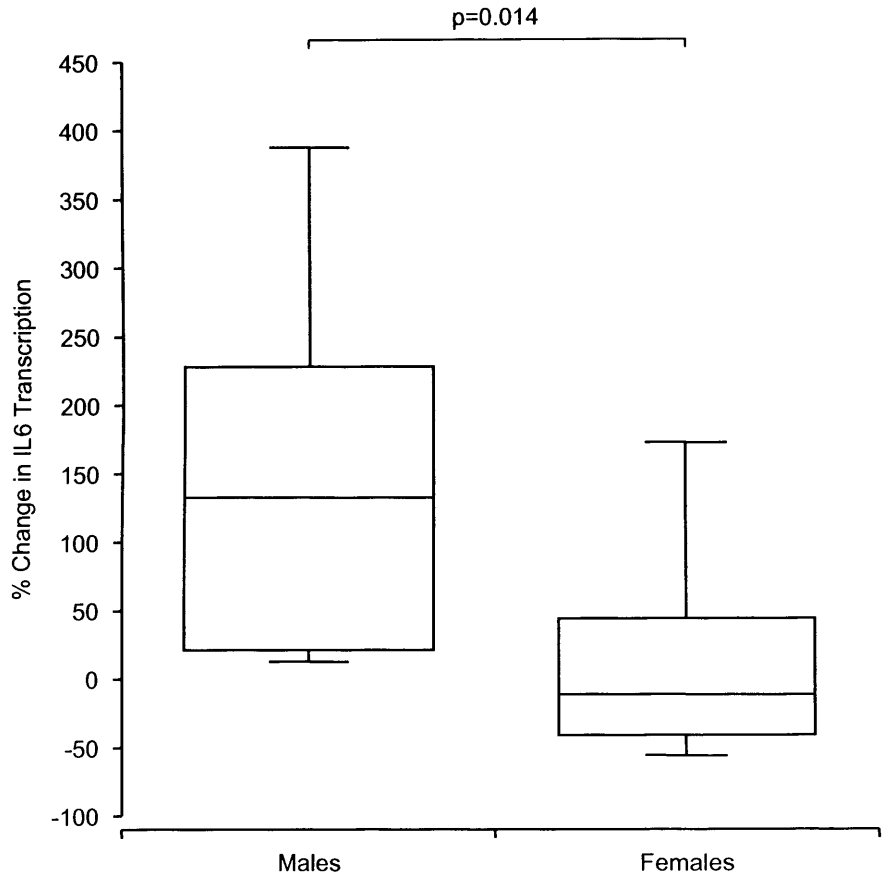


Figure 5.8 Post-operative change in IL6 transcription by gender

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a significantly greater rise in IL6 transcription in males compared to females following surgery (p=0.014, Mann-Whitney U).

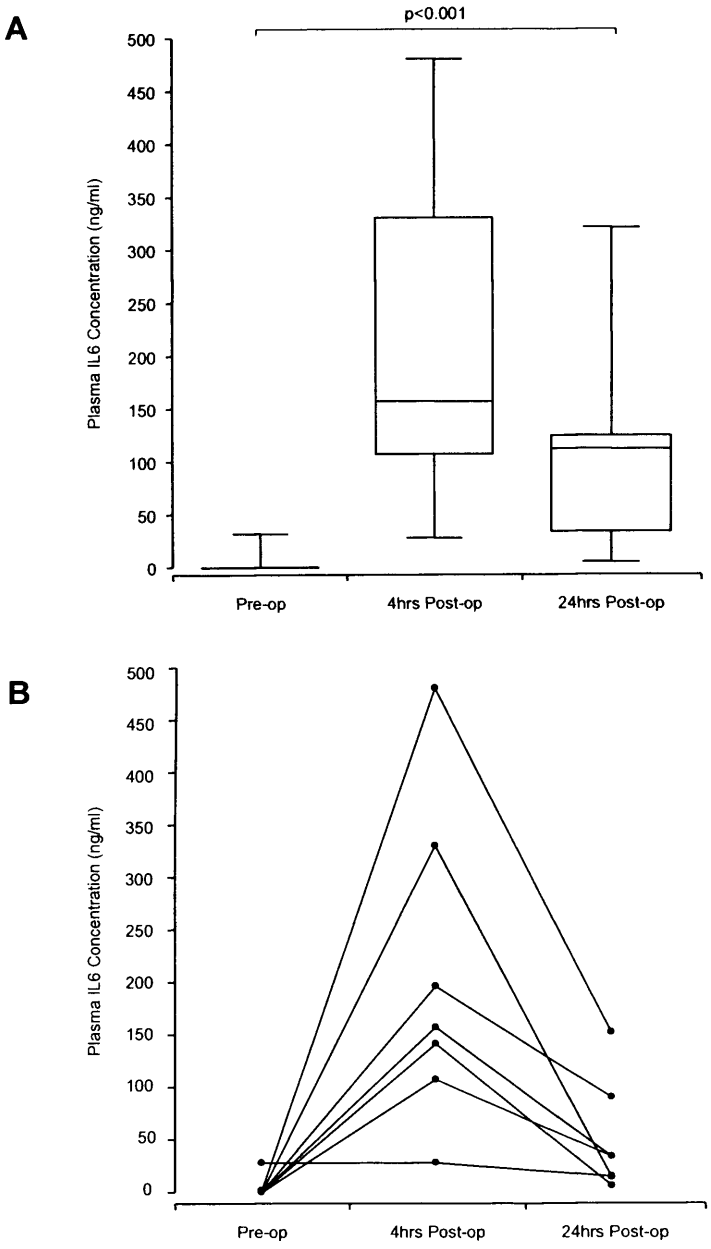


Figure 5.9 Peri-operative plasma IL6 levels

A, Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a definite peri-operative change in IL6 levels ($p<0.001$, Kruskal-Wallis). **B**, Line plot representing results for individual patients with samples available at all 3 peri-operative time points. Each value represents overall grouped mean value, derived from the mean of two separate ELISA experiments.

In the same way that G/C genotype appeared to have no influence on transcription, both post-operative protein levels (p=0.435, Kruskal-Wallis, Figure 5.10) and the change in protein levels (p=0.467, Kruskal-Wallis) also seemed to be unaffected. Similarly, grouping patients according to the presence or absence of a C-allele also revealed no difference in the change in IL6 concentration post-operatively (p=0.526, Mann-Whitney U). Furthermore, in contrast to the gene response, there was no difference between the sexes in terms of post-operative change in IL6 levels (p=0.587, Mann-Whitney U). Once again though, the IL6 plasma protein response to surgery did not correlate with operation time (p=0.240, Spearman's rank correlation), and did not differ depending on the type of procedure undergone (p=0.360, Kruskal-Wallis).

Gene transcription and protein expression

The undetectable pre-operative IL6 plasma protein levels in the majority of patients made it difficult to assess the potential correlation with transcription levels at that timepoint. At the 24 hour post-operative timpoint, there was definite evidence of correlation between IL6 transcription and protein expression (R=0.424, p=0.055, Spearman's rank correlation; Figure 5.11). However, there was no correlation between the gene transcription and protein expression responses to surgery (p=0.997, Spearman's rank correlation).

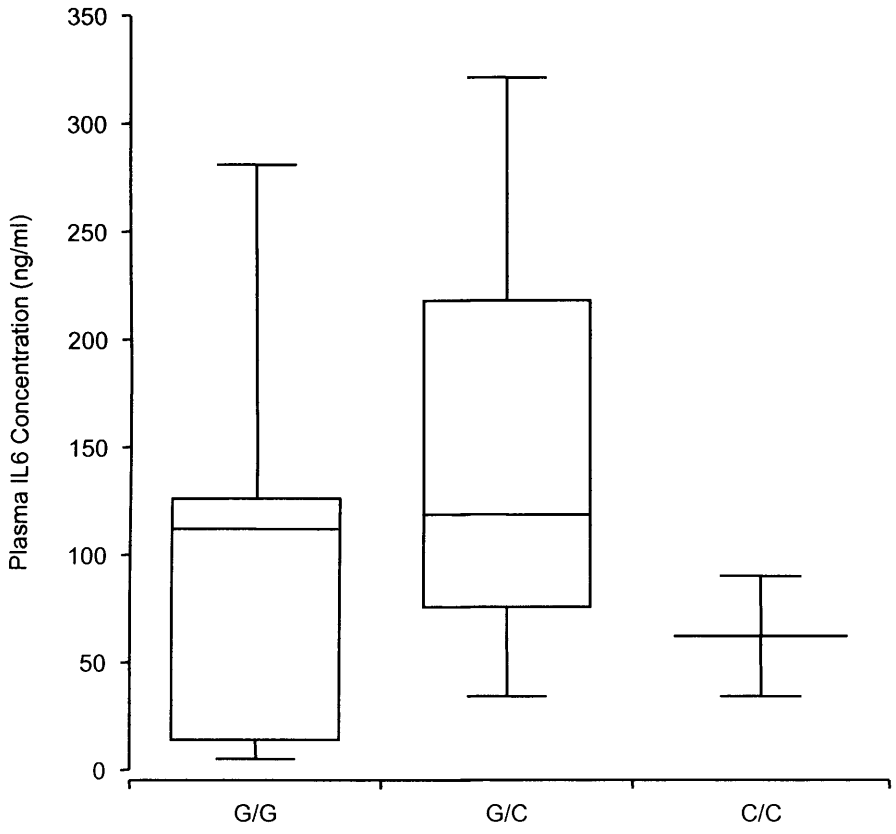


Figure 5.10 Post-operative plasma IL6 levels by G/C genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). Each value represents overall grouped mean value, derived from the mean of two separate ELISA experiments. There was no evidence of a difference between the 3 genotype groups (p=0.435, Kruskal-Wallis).

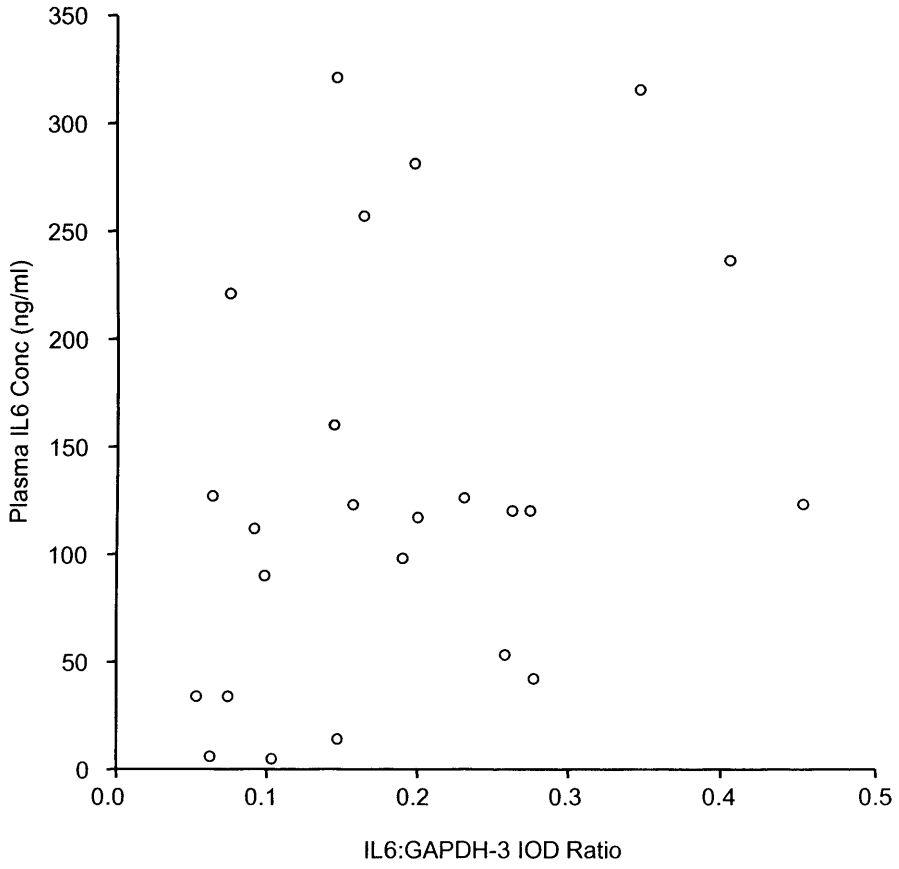


Figure 5.11 Post-operative correlation between IL6 gene transcription and plasma protein expression

Scatter plot representing individual patient results at the 24 hour post-operation timepoint. There was evidence of a correlation between gene transcription and protein expression (R=0.424, p=0.055, Spearman's rank correlation).

5.4 DISCUSSION

The finding of an increase in IL6 protein levels at 24 hours following laparotomy for colorectal cancer is unsurprising given the multifunctional and pro-inflammatory nature of this cytokine. Such a serum protein response to surgery has previously been reported in several studies (Shenkin *et al.,* 1989; Cruickshank *et al.,* 1990; Sarbinowski *et al.,* 2005), and is acknowledged to form part of the inflammatory response to surgery (Biffl *et al.,* 1996). As described in Chapter 1, the extent of this cytokine response has been widely investigated as a being of potential prognostic and diagnostic value. However, peri-operative serum IL6 levels have been found to have varying degrees of significance, and these studies will be discussed in much greater detail in Chapter 7, where clinical outcome is addressed. Nevertheless, a uniform finding amongst these studies was a post-operative rise in IL6, which is in agreement with the results here. As far as the timescale of this rise goes, elevated levels have been detected as early as 2hrs following surgery (Cruickshank *et al.,* 1990), and found to remain elevated up to 72hrs later (Baigrie *et al.,* 1992). Thus, the peak rise found here at 4hrs and prolonged elevation at 24hrs (see Figure 5.9) is in line with past reports (Brull *et al.,* 2001; Chimienti *et al.,* 2006).

These earlier studies have only looked at the serum protein concentrations, without analysing gene transcription. IL6 RNA is known to be both constitutively (Tovey *et al.,* 1988) and inducibly (Steffen *et al.,* 1993) expressed in peripheral blood mononuclear cells. The rapid clearance of circulating IL6 (Castell *et al.,* 1988) suggests that control of plasma concentration lies at the level of transcription; this was reflected by evidence of a rise in transcription levels detected 24hrs following surgery (Figure 5.6). Interestingly, such a gene response to surgery was not detected at the 4hr post-operative

timepoint (Figure 5.5). This may simply reflect the small number of samples available at this interim timepoint, or suggest a lag-time in transcription response. The latter may have been the significant issue, as a 3 hour lag-time in transcription response in IL2-stimulated PBMNs has been described (Panelli *et al.,* 2002). Nevertheless, the combined finding of elevated gene transcription with protein expression 24hrs following colorectal surgery adds validity to the conclusion that a real, rather than spurious, effect was observed. Furthermore, a positive correlation between gene transcription and protein expression at this timepoint was demonstrated (Figure 5.11). This finding partly validates the decision to measure both these as indices of gene expression, and adds weight to the conclusion that a significant post-operative rise in expression did occur. The lack of such an association between the post-operative *changes* in gene transcription and protein expression again probably reflected the difference in response lag-times.

The initial description of the G/C polymorphism in juvenile rheumatoid arthritis patients found that C/C homozygotes had lower levels of protein levels, and gene transcription was reduced in C/C homozygote cell lines (Fishman *et al.,* 1998). However, the opposite effect has also been previously reported, with the C-allele associated with elevated IL6 levels (Jones *et al.,* 2001; Jenny *et al.,* 2002). In this study, there was no evidence of genotypic influence over pre-operative IL6 transcription or protein levels (Figures 5.3 and 5.10). This may reflect an actual lack of effect, or may be due to the small sample size. In addition, limitations in the experimental methodology may have been responsible for the lack of perceived genotypic association, and these will be discussed in turn.

The PCR-based technique of genotyping was a potential source of error. However, this is unlikely, as a well-established method was employed that has previously proved to be accurate (DeMichele *et al.,* 2003), all samples were run twice for confirmation, and the loci were found to be in Hardy-Weinberg equilibrium. The ELISA protein assay could also have been a source of error. This is again unlikely, as once those assays that produced undetectable levels of IL6 were ignored; the intra- and inter-assay co-efficients of variation were within acceptable limits. Nevertheless, the relatively low sensitivity of the assay meant that in the majority (79%) of pre-operative cases IL6 concentration was undetectable, thus making the detection of any genotypic influence difficult. Past work has also reported undetectable levels of IL6 in the majority of pre-operative patients (Bown *et al.,* 2003). In addition, using the same methodology as here, with the cytokine response to surgery defined as the difference between post-operative and induction concentrations, and non-detectable levels counted as zero, no allelic association of IL6 levels was detected either. Similarly, this independence from genotype of IL6 levels has also previously been reported in a larger study of pre-operative CABG patients (Brull *et al.,* 2002). There has only been one study published, looking at pre-operative colorectal cancer patients, and even using an ultra-sensitive assay with the ability to detect concentrations as low as 0.1pg/ml, there was still no genotypic influence on IL6 protein levels (Belluco *et al.,* 2002). However, by grouping their patients into the presence (G/C and C/C) or absence (G/G alone) of a C-allele, a significant influence was found; patients possessing a C-allele had lower protein levels (Belluco *et al.,* 2002).

With gene transcription, once again, the apparent lack of genotypic influence may be due to the choice of a semi-quantitative RT-PCR technique. Recent advances in

molecular biology technology have led to the introduction of the quantitative "real-time" technique for measuring levels of gene transcription. This method involves the collection of data throughout the PCR process, thereby combining the amplification and detection into a single step. Thus, PCR product concentration is correlated to fluorescence intensity, allowing the gathering of quantitative data. However, the great sensitivity of real-time PCR does mean that rigorous normalisation is required before accurate conclusions can be made, and differences in primer efficiency can affect transcription results (Wong *et al.,* 2005). Furthermore, the great cost of the expensive equipment and reagents required for real-time PCR is often prohibitive. In addition, the semi-quantitative RT-PCR has previously been demonstrated to be comparable to real-time in determining changes and relative differences in gene transcription (Peirson *et al.,* 2005). Alternatively, the lack of genotypic association could be attributed to the presence of only four C/C homozygotes in the study population.

Employing the same allele-breakdown analysis as described earlier (Belluco *et al.,* 2002) to pre-operative transcription levels, failed to produce significant findings; there was no evidence of allelic influence on pre-operative transcription. It may be that this was due to the complexity of control, due to the existence of a multitude of IL6 polymorphisms, means one should look at haplotypes rather than an individual SNP such as -174 G/C in isolation (Terry *et al.,* 2000). In addition, this may have combined with the wide variety of possible stimulating factors, and the plethora of past antigenic exposure in adult subjects, to make it difficult for the influence of a single functional polymorphism over IL6 expression to be accurately observed; thus in neonates, with their relatively naïve immune system, a genotypic influence on IL6 levels has been observed, compared to no such impact in healthy adults (Kilpinen *et al.,* 2001).

Post-operatively, in this cohort, genotype seemed to exert more influence on IL6 transcription, but no impact on protein levels was observed. A definite trend was observed towards C/C homozygotes having reduced IL6 RNA transcripts (Figure 5.4). This finding in human PBMN cells is in agreement with, and extends the original description of the -174 G/C polymorphism, which reported results from cell lines (Fishman *et al.,* 1998), and may reflect the fact that both post-operative and juvenile chronic arthritis patients are subject to an inflammatory stimulus. However, it is difficult to place this finding into context, since despite there being published studies investigating IL6 following surgery, these have concentrated on plasma IL6 protein levels, and none have examined gene transcription. In fact, when examining such studies, inconsistent conclusions become evident. In AAA patients, where the aneurysm itself acts as an inflammatory source of circulating IL6, the C allele was associated with elevated plasma IL6 levels (Jones *et al.,* 2001). Similarly, previous investigation of CABG patients has reported a trend towards C/C homozygotes having greater post-operative IL6 protein levels compared to G-allele carriers, only becoming significant when possible confounding factors such as smoking-status, operation time and body mass index were taken into account (Brull *et al.,* 2001).

Conversely, more recent studies of CABG patients concluded that G/G homozygotes had greater post-operative IL6 protein levels (Burzotta *et al.,* 2001; Gaudino *et al.,* 2003), although rather than look at just the 24hr timepoint, the total IL6 response up to the time of discharge was measured. Interestingly, the significance of possible confounding factors also remains unclear. Intra-operative factors such as procedure length and transfusion of blood products have been shown to have no impact over the cytokine response to surgery (Bown *et al.,* 2003), whereas others have found a score of

operative severity to be of influence (Chimienti *et al.,* 2006). In fact, this latter study concluded that the IL6 response to major abdominal surgery was predictable from a linear combination of operative severity and IL6 genotype, except this time, the C-allele associated with low IL6 plasma protein levels at 24hrs post-operation (Chimienti *et al.,* 2006). Even so, the tendency demonstrated in this study for C/C homozygotes to have lower levels of gene transcription following surgery would seem to favour those studies that reported similar results, but with protein levels.

Turning to the scale of the protein and transcription response to surgery, rather than just post-operative levels, in this study both of these seemed to be independent of G/C genotype (Figure 5.7). The only other investigation of IL6 response, as defined by the difference between post-operative and pre-operative baseline samples, only measured plasma protein levels, and also failed to demonstrate any genotypic influence in patients undergoing AAA repair (Bown *et al.,* 2003). Furthermore, allele-based grouping still did not reveal any significant differences. Thus the demonstration of a lack of genotypic influence over the IL6 transcription response extends previous findings. Once again, this may be a true result, or could be due to the same experimental issues discussed earlier. Alternatively, it may be that the timecourse of the IL6 response rather than the peak extent is of physiological importance and under genotypic control; the choice of only two post-operative sampling timepoints did not allow proper assessment of this.

The investigation of gender-related cytokine response has been carried out, prompted by clinical studies that found a preponderance of morbidity and mortality in males compared to females following major surgery (Katz *et al.,* 1997; Offner *et al.,* 1999).

Despite this not being a uniform finding, with women being found to fare worse following burns trauma (O'Keefe *et al.,* 2001), the concept of a gender-specific immune response has been examined. So, in patients undergoing colonic surgery, women have been found to have higher serum IL6 protein levels on post-operative day 1 (Wichmann *et al.,* 2003). However, this study may have been compromised by poor homogeneity of the patient population, with some undergoing oncological surgery, and others having pre-operative chemotherapy and radiation. Indeed more recent examination of patients undergoing pancreatic surgery failed to demonstrate any gender-specific differences in IL6 protein levels post-operatively (Scheingraber *et al.,* 2005). Then again, these pancreatic patients also underwent a wide spectrum of procedures, differing greatly in severity, making a valid gender-based comparison difficult. Nevertheless, this lack of gender-influence on post-operative IL6 protein levels was also observed in this study, which examined a more uniform subject population. On the other hand, the finding that males launched a greater IL6 gene transcription response to surgery compared to females has not previously been reported (Figure 5.8). Interestingly, given that raised IL6 post-operative levels (Mokart *et al.,* 2005) and male gender (Offner *et al.,* 1999) have both been found to correlate with subsequent sepsis, this observation of a greater male transcription response to surgery may be significant. The possibility of such an association with outcome will be discussed in greater detail in Chapter 7.

Finally, the scale of the IL6 plasma protein response to surgery, assessed mostly by measuring the peak value, has previously been correlated with the duration and severity of the operation (Shenkin *et al.,* 1989; Baigrie *et al.,* 1992), with the difference between laparoscopic and open approaches of particular interest (Hildebrandt *et al.,* 2003). This was not the case in this study, with the finding that neither the IL6 gene transcription

nor protein expression response to surgery correlated with operation time. Such a lack of association may have been due to the experimental issues previously discussed. It is more likely that the 24 hour post-operative sampling time may have been too late to detect any association with operative severity, as IL6 plasma levels would have peaked earlier at around 6 hours (Shenkin *et al.,* 1989). Unlike plasma protein concentrations, the timecourse of the transcription response has not been established in surgical patients, and it may be that a lag-time in this gene reaction may be responsible for the lack of association with operation time found at 24 hours.

To summarise the main findings of this investigation of the peri-operative IL6 response to surgery:

- A rise in both IL6 protein levels and gene transcription was observed 24 hours following surgery (Figures 5.6 and 5.9).
- There was no evidence of an IL6 -174 G/C genotypic association with IL6 protein or gene transcription levels at the baseline pre-operative timepoint (Figures 5.3 and 5.10).
- Post-operatively, there was a tendency for IL6 -174 C/C homozygotes to have reduced levels of gene transcription, although no such association with protein levels was seen (Figures 5.4 and 5.11).
- The IL6 protein expression and gene transcription responses to surgery were not associated with IL6 -174 G/C genotype or operative duration.
- Males launched a greater IL6 gene transcription response to surgery compared to females (Figure 5.8), although this was not reflected in the plasma protein response.

Having addressed the gene expression response to surgery, and the influence of a common genetic polymorphism, for one of the most important cytokines, IL6, attention will be turned towards a similar analysis of the increasingly investigated pro-inflammatory ACE.

CHAPTER 6

Surgery ACE I/D Genotype and Expression

CHAPTER 6

6.1 INTRODUCTION

ACE a key component of the renin-angiotensin system, involved in maintaining fluid homeostasis during hypovolaemic challenges. However, as well as this, there is a growing body of evidence pointing to a role for ACE as a pro-inflammatory cytokine, able to upregulate all stages of the acute inflammatory response (Cheng *et al.,* 2005; Drexler *et al.,* 1995; Potter *et al.,* 1998). Given that the trauma of surgery can often result in a state of hypovolaemia, and also provokes an initial pro-inflammatory cytokine response (Baumann *et al.,* 1994), it is reasonable to propose that ACE expression may be altered in the post-operative phase.

The discovery of the functional I/D polymorphism within intron 16 of the ACE gene (Rigat *et al.,* 1990) established a plausible genetic basis for the wide inter-individual variation in ACE levels observed. Subsequently, possession of the D-allele was associated with higher circulating plasma ACE levels (Costerousse *et al.,* 1993), and more recent investigation of I/D heterozygotes demonstrated the D-allele to be responsible for greater gene transcription than the I-allele (Suehiro *et al.,* 2004). Consequently, much interest has concentrated on the possible association between this polymorphism and a whole host of disease states, with the majority of the diseases chosen for investigation being inflammatory or cardiovascular in nature. To date, only one study has investigated the role of the I/D polymorphism in influencing outcome from surgery (Lee *et al.,* 2005); this focused solely on oesophagectomy patients and concluded that the D-allele was associated with a greater risk of developing pulmonary complications. In this study here, the effect of surgery for colorectal cancer on ACE is examined, in terms of both plasma ACE levels and gene transcription. Furthermore, the

differential effect of the inflammatory insult of surgery on the individual I and D-alleles is investigated.

6.2 MATERIALS AND METHODS

As described in Chapter 3, following ethical approval and informed consent, blood samples were taken from elective colorectal cancer surgery patients pre-operatively and, when possible, 4 hours, 24 hours, 10 weeks and 20 weeks following surgery. PBMNs and plasma were separated from blood by a process of centrifugation through a density gradient polymer and from the former, nucleic acids extracted using a phenol-ethanol precipitation method (see Sections 3.2 and 3.3).

Gene transcription

An RT reaction was performed on the extracted RNA to produce cDNA (Section 3.6). Primers specific for the ACE gene were designed, and a PCR used to amplify mRNA transcripts, so that a semi-quantitative method of measuring gene transcription could be used (Section 3.6). These PCR products were separated by agarose gel electrophoresis, and analysed by scanning densitometry. Results were normalised by comparing transcription to that of the standard house-keeping gene GAPDH-3 (Section 3.6).

An analysis of the differential expression of the individual alleles was achieved in the ACE I/D heterozygote patients by exploiting the direct and consistent linkage between the intronic I/D polymorphism and the exonic G2215A SNP, which is subsequently transcribed into the mRNA sequence; the I-allele is always found in concert with the 2215G-allele (Section 3.7). An overall comparison of allele activity was made by

grouping the contribution from the D-allele from the heterozygotes with that of the DD homozygotes, and the I-allele with the II homozygotes.

Each sample was run twice, and the final results correspond to the mean of these two experiments. In the case of overall ACE transcription, this was derived from the mean of the experiments using the specifically designed ACE216 primers (Section 3.6) and the ACE2215 primers used when assessing differential allelic contribution (Section 3.7).

Plasma protein expression

Levels of ACE protein were assessed using a commercially available ELISA kit (Section 3.8). Samples were run in duplicate, and the final result representing the mean of two separate experimental plates. An estimation of experimental accuracy was made by evaluating the inter- and intra-experimental variation; in this case, for ACE, the co-efficient of variation was 7% for intra-assay determinations, and 5% for inter-assay determinations.

6.3 RESULTS

Gene transcription

The patient demographics correspond to those discussed in Chapter 4. Due to difficulties with patient recruitment and compliance, limited samples were available at the 4hr post-operative time point. A representative picture of an agarose gel with RT-PCR products for the assessment of ACE transcription is shown in Figure 6.1. Raw results of gene transcription are presented in Appendix IV. Analysis of these with the Kolmogorov-Smirnov test for normality demonstrated values to be normally distributed.

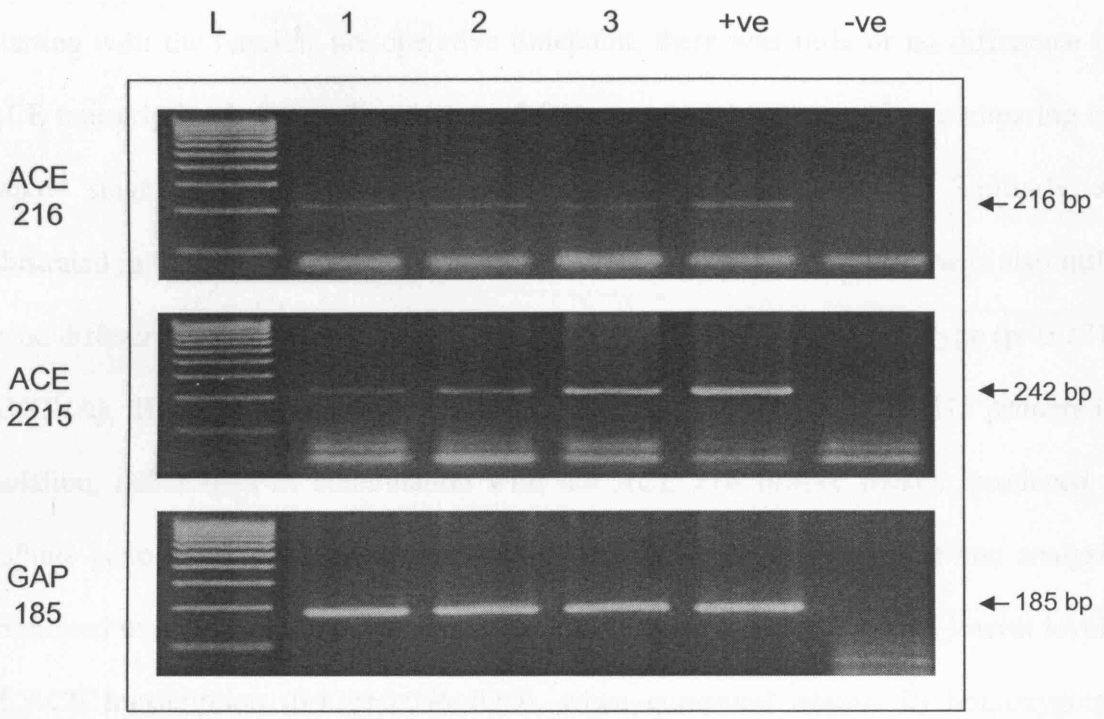


Figure 6.1 ACE gene transcription determined by RT-PCR

Representative picture of ACE 216 and ACE 2215 PCR products. L, 100bp molecular reference ladder; 1-3, patient samples; +ve, positive control; -ve, negative control. GAPDH-3 used as internal control for the PCR reaction; respective gene transcript sizes (bp) are indicated.

Statistical analysis was thus performed using parametric tests and graphically, results are presented as mean values ± s.e.m.

Pre-operative timepoint

Starting with the baseline pre-operative timepoint, there was little or no difference in ACE transcription between the sexes (p=0.504, unpaired t-test), or when comparing by Dukes' stage (p=0.153, ANOVA) or ASA score (p=0.329, ANOVA). Similarly, as illustrated in Figure 6.2, despite the graphical suggestion of a trend, there was also little or no difference when analysing pre-operative ACE transcription by genotype (p=0.271, ANOVA). However, analysing the transcription results using the ACE 2215 primers in isolation, rather than in combination with the ACE 216 primer results, produced a definite genotypic pattern (p=0.023, ANOVA), see Figure 6.3, and post-hoc analysis confirmed that, with the ACE 2215 primer set, D/D homozygotes had the lowest levels of ACE transcription (0.172±0.046 IOD), when compared against I/I homozygotes (0.422±0.049 IOD, p=0.035, Bonferroni's) and I/D heterozygotes (0.380±0.052 IOD, p=0.046, Bonferroni's).

24 hour post-operative timepoint

The situation 24 hours post-operation was no different, with there again being no difference in ACE transcription when comparing by sex (p=0.539, unpaired t-test), Dukes' stage (p=0.242, ANOVA), ASA score (p=0.397, ANOVA) or ACE genotype (p=0.474, ANOVA). In contrast to pre-operative samples however, the further breakdown analysis of the results from the ACE 2215 polymorphism primers alone did not yield any differences.

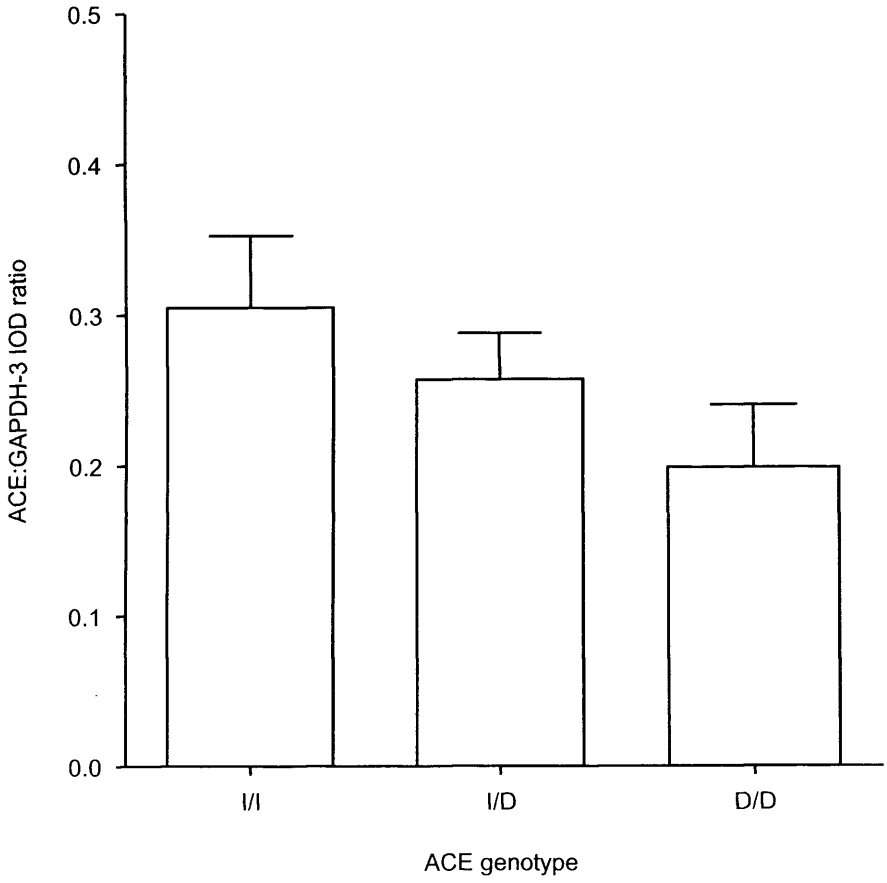


Figure 6.2 Pre-operative ACE transcription by genotype

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. Despite the appearance of a trend, there was no significant difference in pre-operative ACE transcription between the genotypes ($p=0.271$, ANOVA).

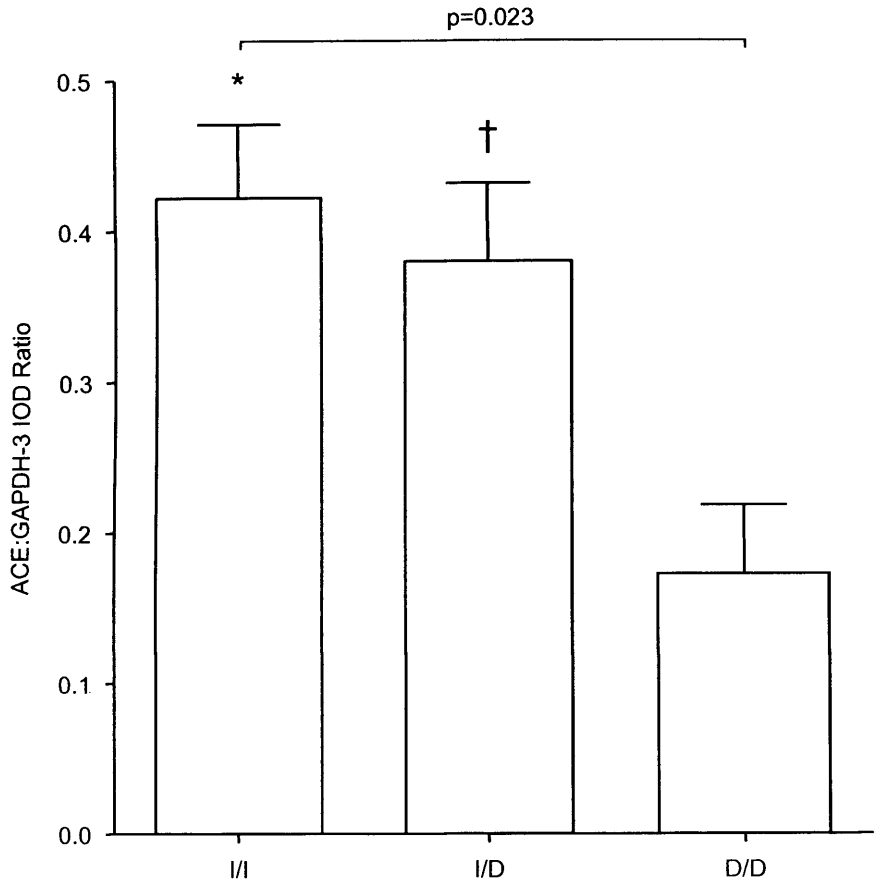


Figure 6.3 Pre-operative ACE 2215 transcription by genotype

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. With ACE 2215 primers, there was significant difference in pre-operative ACE transcription between the genotypes (p=0.023, ANOVA). * Post-hoc analysis demonstrated that I/I homozygotes had significantly greater transcription levels compared to D/D patients (p=0.035, Bonferroni's). † Similar finding for I/D patients (p= 0.046, Bonferroni's).

I/D allele breakdown

The differential contribution of the individual I- and D-alleles, was analysed in the subset of 12 I/D heterozygotes. A representative picture of the RT-PCR and HAE II restriction enzyme reaction is presented in Figure 6.4. Pre-operatively, see Figure 6.5, there was a definite increased contribution of the D-allele compared to the I-allele, to overall ACE gene transcription (0.335±0.049 *vs* 0.134±0.025 IOD, $p<0.001$, paired t-test). Indeed, on average pre-operatively, the D-allele produced 3.25±0.34 times the number of RNA transcripts compared to the I-allele (Figure 6.6). The situation at 24 hours post-operation was unchanged for this cohort of 12 heterozygotes, with the D-allele again dominating the ACE transcription (0.359±0.077 *vs* 0.171±0.026 IOD, $p=0.010$, paired t-test); its contribution to transcription was on average 2.37±0.29 times that of the I-allele (Figures 6.7 and 6.8).

The separate allelic contributions for the study population as a whole were analysed, by combining this heterozygote breakdown with transcription results, using the same ACE 2215 primers, from homozygotes. Pre-operatively, there was a definite trend towards the D-allele (combination of D/D homozygotes and D-allele contribution of heterozygotes) being responsible for greater levels of ACE gene transcription than the I-allele (combination of I/I homozygotes and I-allele contribution of heterozygotes) (0.289±0.038 *vs* 0.197±0.030 IOD, $p=0.06$, unpaired t-test, Figure 6.9). Post-operatively, D-allele transcription again seemed to predominate, but now to a much lesser extent (0.337±0.055 *vs* 0.240±0.035 IOD, $p=0.15$, unpaired t-test, Figure 6.10).

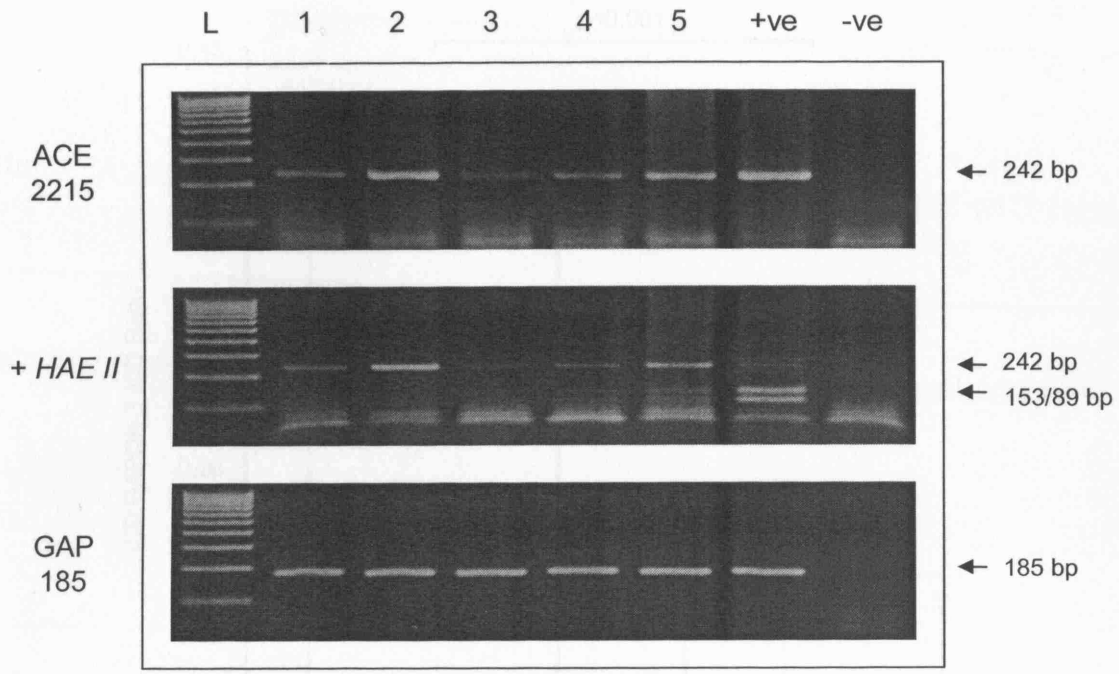


Figure 6.4 Differential ACE allele transcription

Representative picture of ACE 2215 PCR products and HAE II digestion products. L, 100bp molecular reference ladder; 1-5, patient samples; +ve, positive control; -ve, negative control. GAPDH-3 used as internal control for the PCR reaction; respective gene transcript sizes (bp) are indicated.

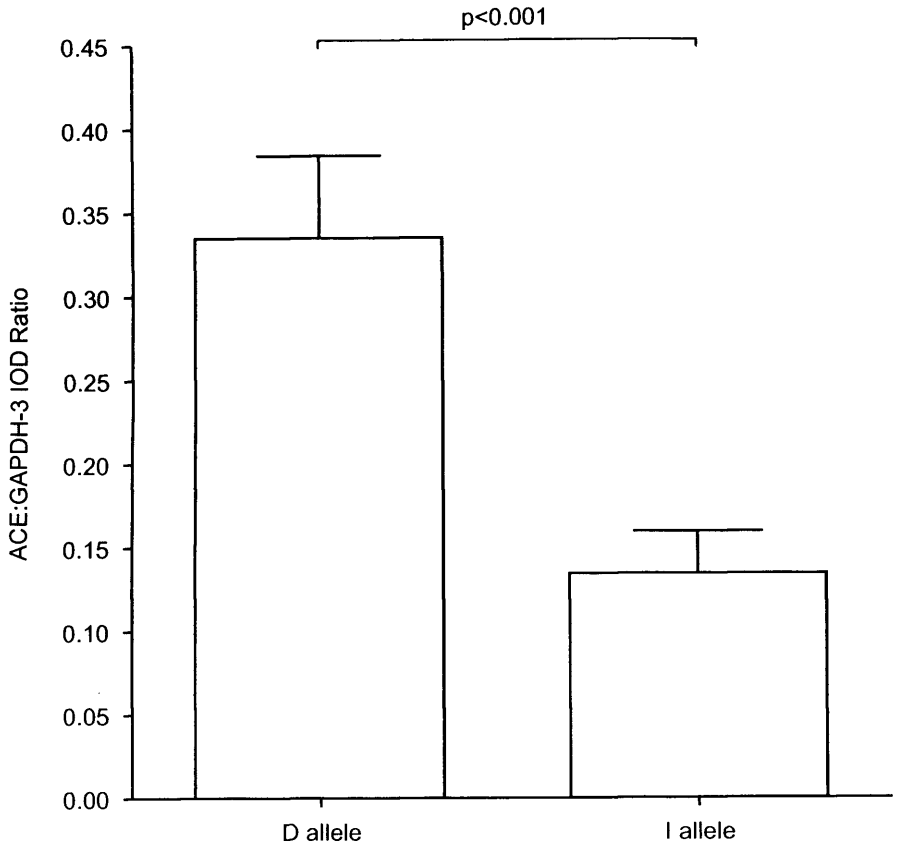


Figure 6.5 Pre-operative allele breakdown of ACE transcription in I/D heterozygotes

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. Pre-operatively, the D-allele produced significantly greater transcription than the I-allele ($p<0.001$, paired t-test).

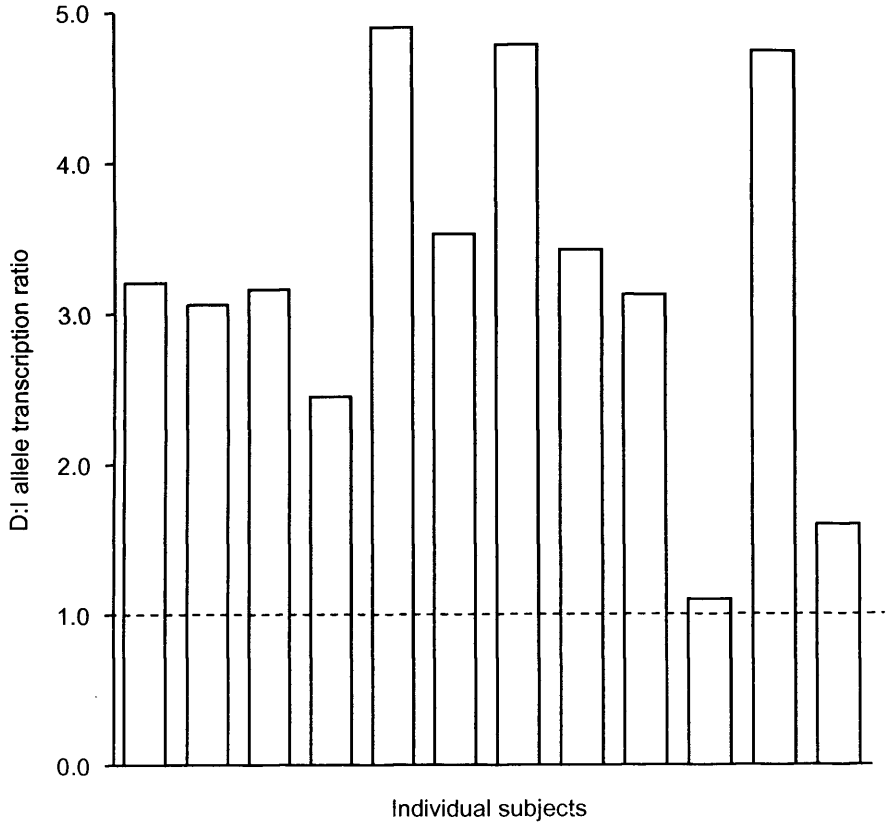


Figure 6.6 Pre-operative ratios of D:I allele transcription in I/D heterozygotes

Bars represent the ratio of D:I allele transcription for each I/D heterozygote subject. The dashed line denotes the ratio of 1 – when alleles produce equal transcription. All the subjects had a ratio > 1, with a mean ratio of 3.25±0.34.

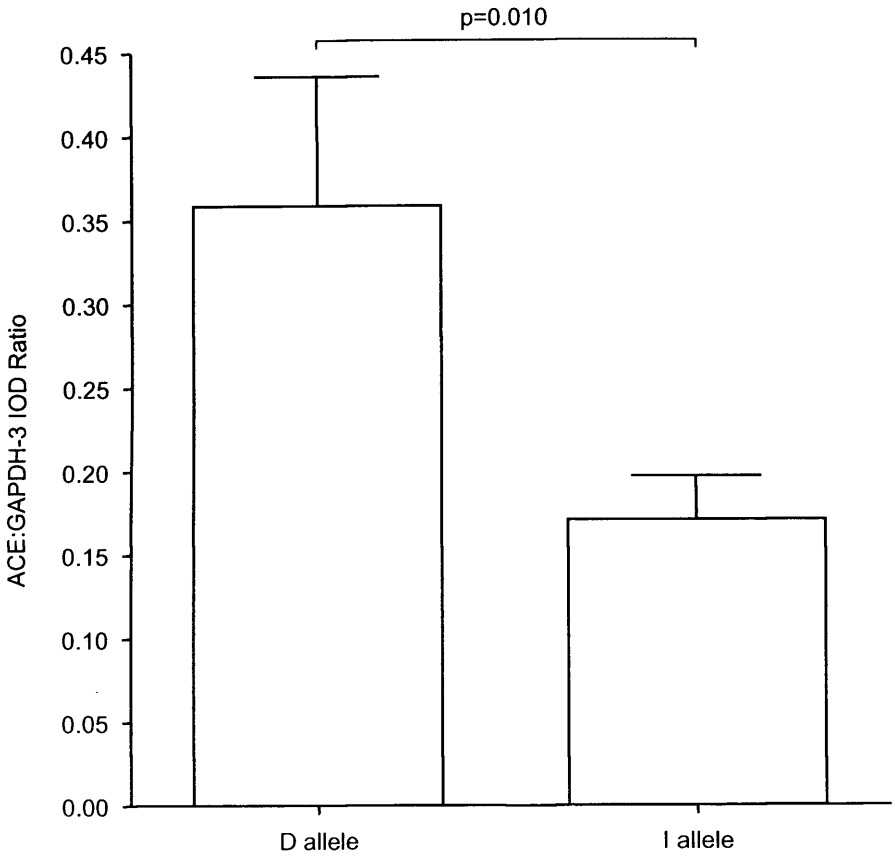


Figure 6.7 Post-operative allele breakdown of ACE transcription in I/D heterozygotes

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. Post-operatively, the D-allele still produced significantly greater transcription than the I-allele (p=0.010, paired t-test).

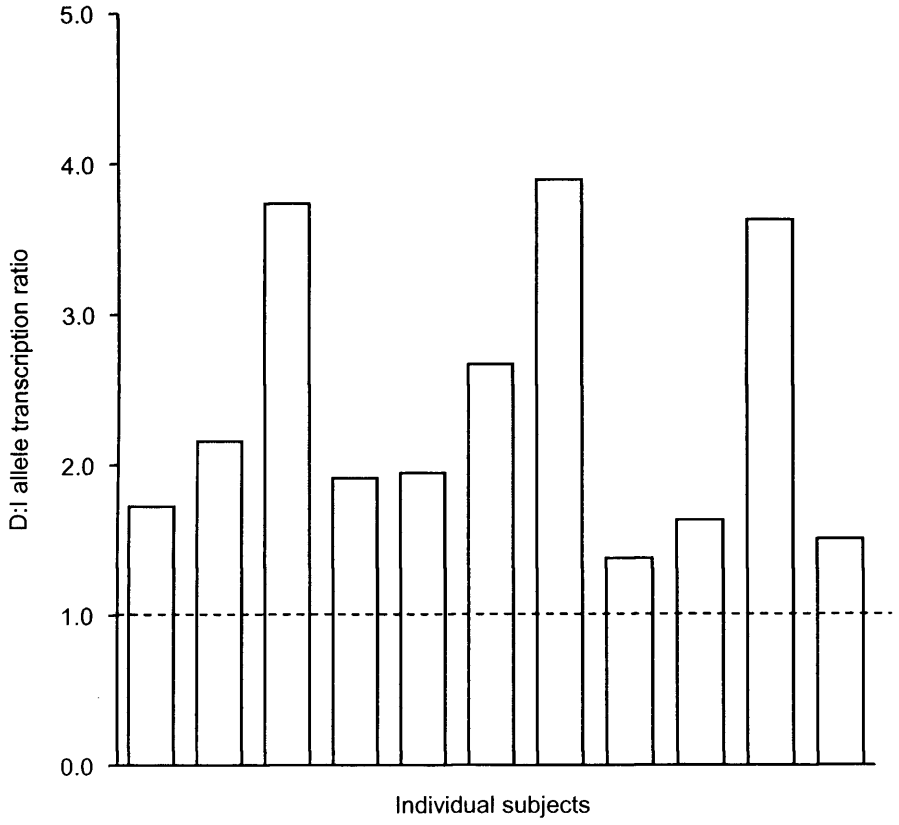


Figure 6.8 Post-operative ratios of D:I allele transcription in I/D heterozygotes

Bars represent the ratio of D:I allele transcription for each I/D heterozygote subject. The dashed line denotes the ratio of 1 – when alleles produce equal transcription. Again, all the subjects had a ratio > 1, with a mean ratio of 2.37±0.29.

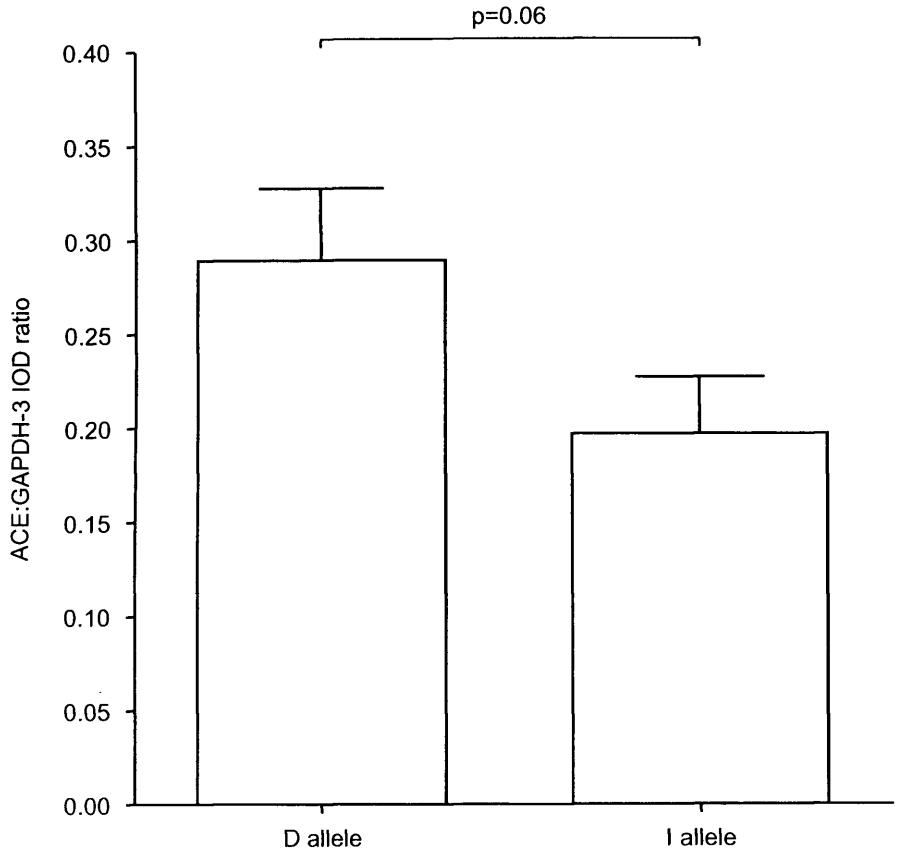


Figure 6.9 Pre-operative allele breakdown of ACE transcription for whole study population

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. The D-allele tended to produce greater transcription than the I-allele (p=0.06, unpaired t-test).

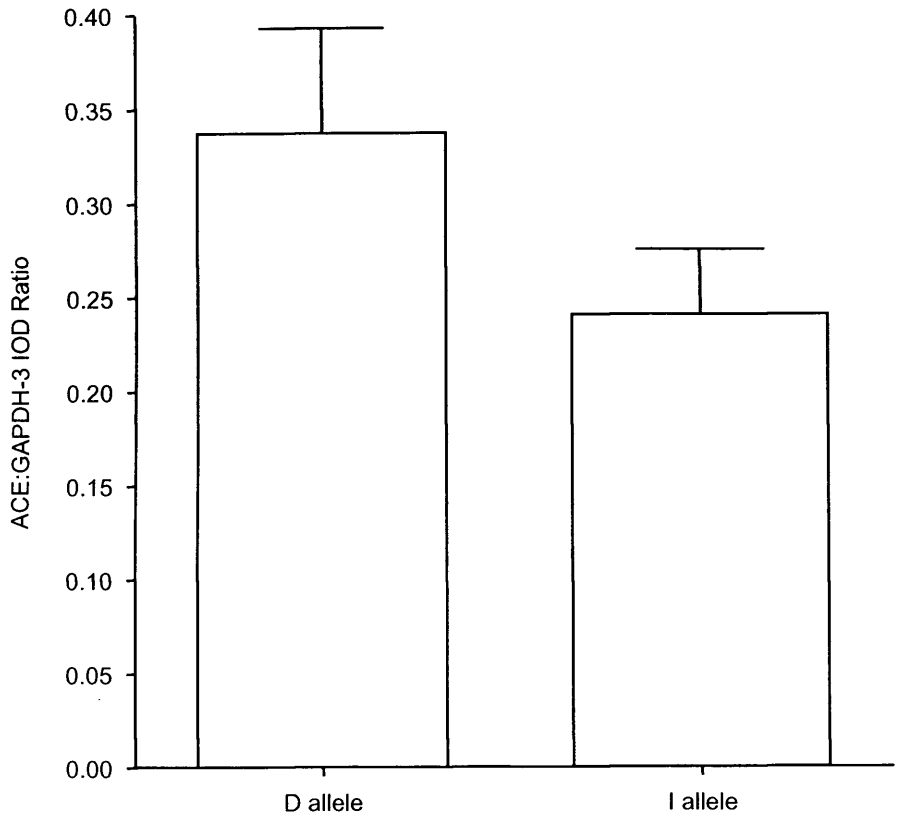


Figure 6.10 Post-operative allele breakdown of ACE transcription for whole study population

Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses. The D-allele still seemed to produce greater transcription than the I-allele, but to a much lesser degree ($p=0.15$, unpaired t-test).

Peri-operative ACE transcription response

Analysing the peri-operative period, including the 4-hour and 24-hour timepoints, surgery appeared to have a definite effect on ACE gene transcription ($p=0.064$, ANOVA), see Figure 6.11. In fact when discounting the 4 hour timepoint, and just comparing the pre- and 24 hour post-operative situations, there was a significant rise in ACE transcription (0.256 ± 0.022 *vs* 0.332 ± 0.029 IOD, pre-op *vs* post-op, $p=0.042$, unpaired t-test). Moreover, if transcription results using the ACE 2215 primers in isolation, rather than in combination with the ACE 216 primer results were again analysed, this post-operative rise was even more evident (0.345 ± 0.036 *vs* 1.186 ± 0.098 IOD, pre-op *vs* post-op, $p<0.001$, unpaired t-test, Figure 6.12).

Results for percentage change in transcription failed the Kolmogorov-Smirnov test for normality, prompting the presentation of data as median (range) and the use of non-parametric tests. Analysis of whether the scale of the rise in ACE gene transcription, as measured by percentage change, was dependent on ACE genotype, revealed no such relationship ($p=0.643$, Kruskal-Wallis). However looking at the results of the change in gene transcription from the ACE 2215 primers in isolation, a definite genotypic pattern emerged (65 (0-265) *vs* 302 (105-703) *vs* 812 (548-1545) %, I/I *vs* I/D *vs* D/D, $p=0.002$, Kruskal-Wallis, Figure 6.12) with D/D homozygotes mounting the greatest response to surgery.

Turning to the response of the individual alleles to the stimulus of surgery, in the subset of I/D heterozygotes, there was initially no evidence of any preferential allelic activation.

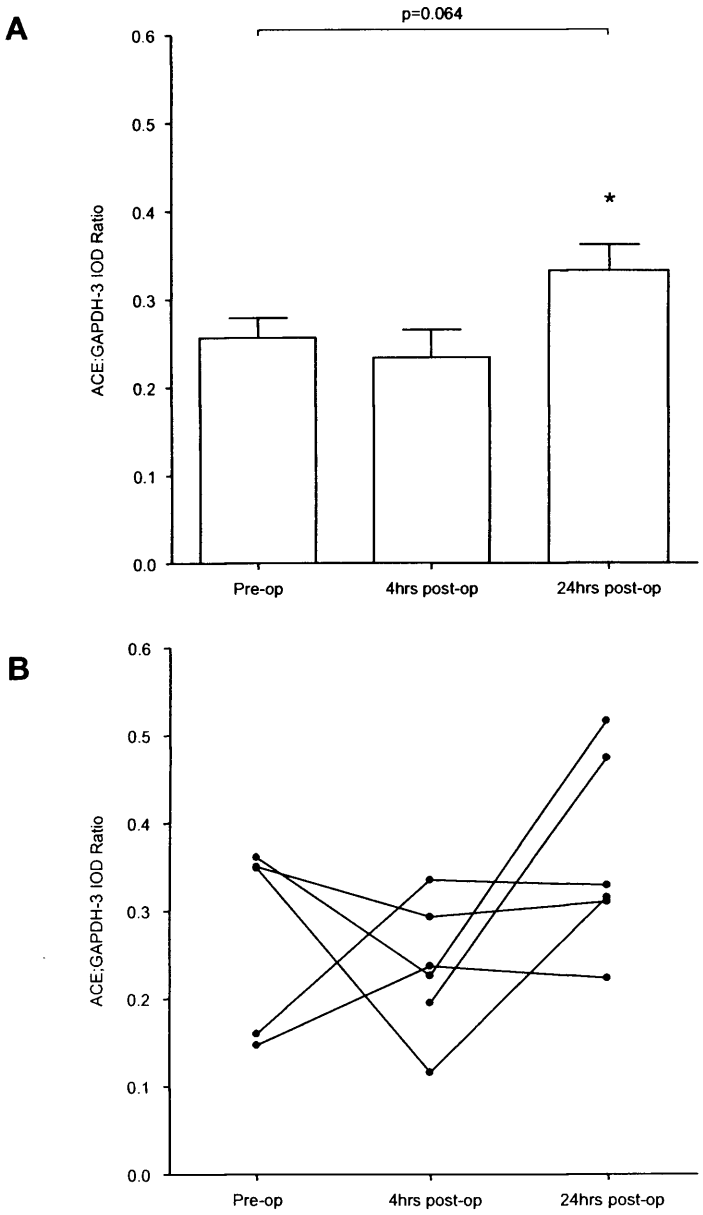


Figure 6.11 Peri-operative change in ACE transcription

A, Bars represent mean values, errors are s.e.m. There was a definite trend towards difference between genotypes in change ACE transcription following surgery (p=0.064, ANOVA). * Looking at just the pre-op and 24hrs post-op timepoints, transcription was elevated (p=0.042, unpaired t-test). **B**, Line plot representing results for individual patients with samples available at all 3 peri-operative time points. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses.

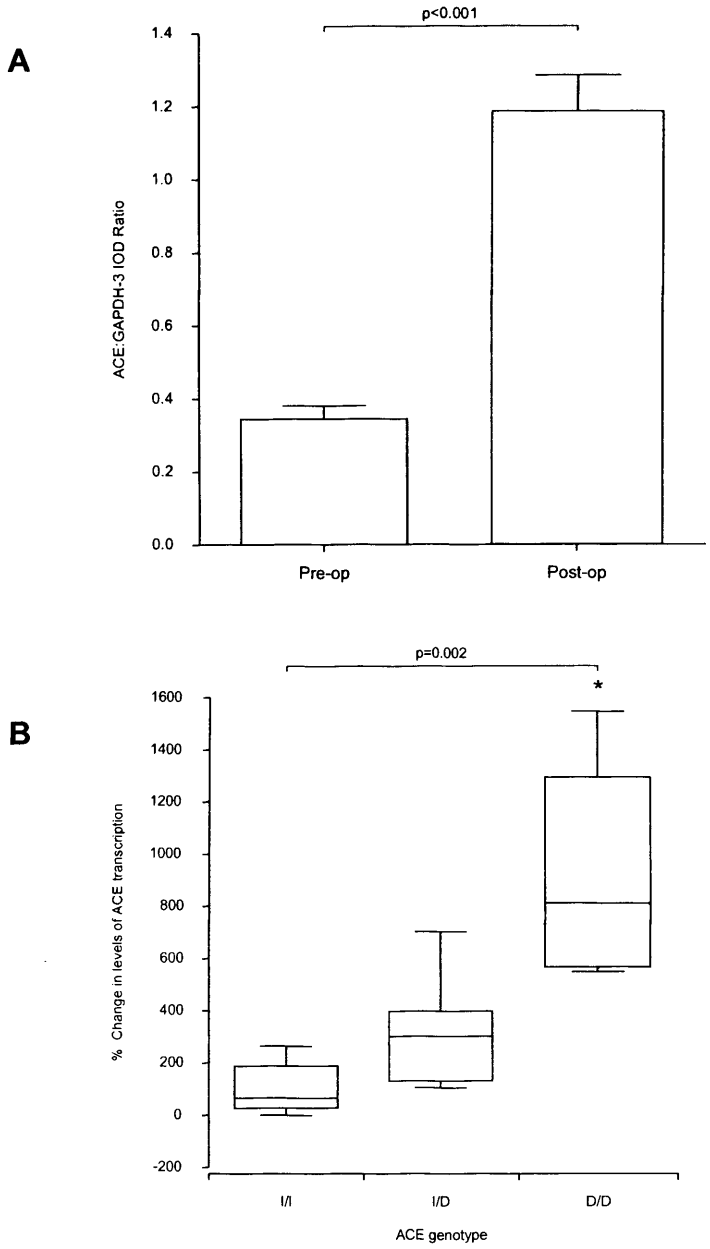


Figure 6.12 Post-operative change in ACE transcription and I/D genotypic influence with 2215 primers

A, Bars represent mean values, errors are s.e.m. IOD, total integrated optical density of ACE:GAPDH-3. There was a significant rise in ACE gene transcription following surgery ($p<0.001$, Mann-Whitney U). **B**, Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a definite association between ACE genotype and the percentage change in ACE transcription following surgery, with D/D homozygotes launching the greatest response ($p=0.002$, Kruskal-Wallis).

Transcription from the D-allele did not significantly alter from before surgery to afterwards (0.335±0.049 *vs* 0.359±0.077 IOD, p=0.760, paired t-test). Similarly, transcription originating from the I-allele did not change following surgery (0.134±0.025 *vs* 0.171±0.026, pre- *vs* post-operation, p=0.247, paired t-test). However, there was a significant fall in the ratio of D:I allele transcription when comparing the pre- and post-operative states (3.10±0.34 *vs* 2.38±0.29), p=0.034, paired t-test, Figure 6.13), suggesting an alteration in the relative contribution, towards greater I-allele input.

Plasma protein expression

Moving on to the ACE plasma levels, the raw results from the ELISA experiments are presented in Appendix V. Once again, analysis with the Kolmogorov-Smirnov test revealed that the values for levels of plasma ACE were normally distributed, prompting the further use of parametric statistical tests, and results being presented graphically as mean values ± s.e.m.

Pre-operatively, despite there being no definite difference when comparing by genotype (p=0.298, ANOVA, Figure 6.14), there was a slight suggestion that I/I homozygotes might have lower levels of plasma protein expression (173±12 *vs* 179±8 *vs* 199±16 ng/ml, I/I *vs* I/D *vs* D/D genotypes).

There was a definite trend towards the females having higher levels of ACE protein expression (196±12 *vs* 174±9 ng/ml, p=0.080, unpaired t-test). At the 24 hour post-operative timepoint, the situation was no different, with the ACE genotype again having no definite influence on levels (p=0.422, ANOVA, Figure 6.15).

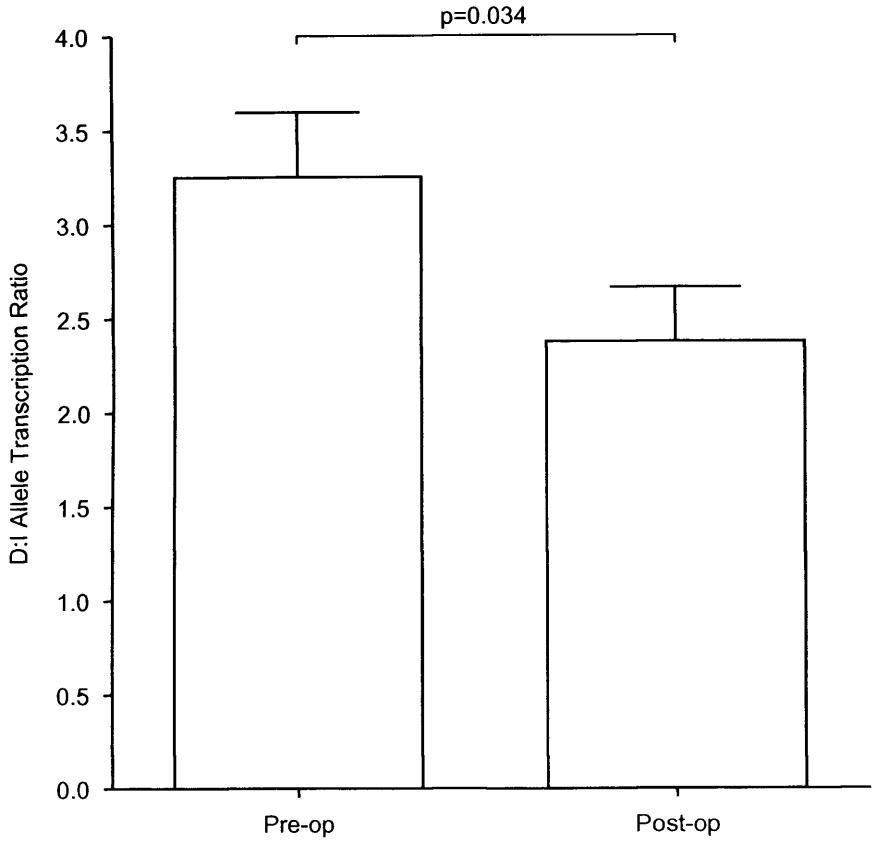


Figure 6.13 Peri-operative change in D:I allele ratio of transcription

Bars represent mean values, errors are s.e.m. There was a significantly lower D:I ratio post-operatively (p=0.034, paired t-test).

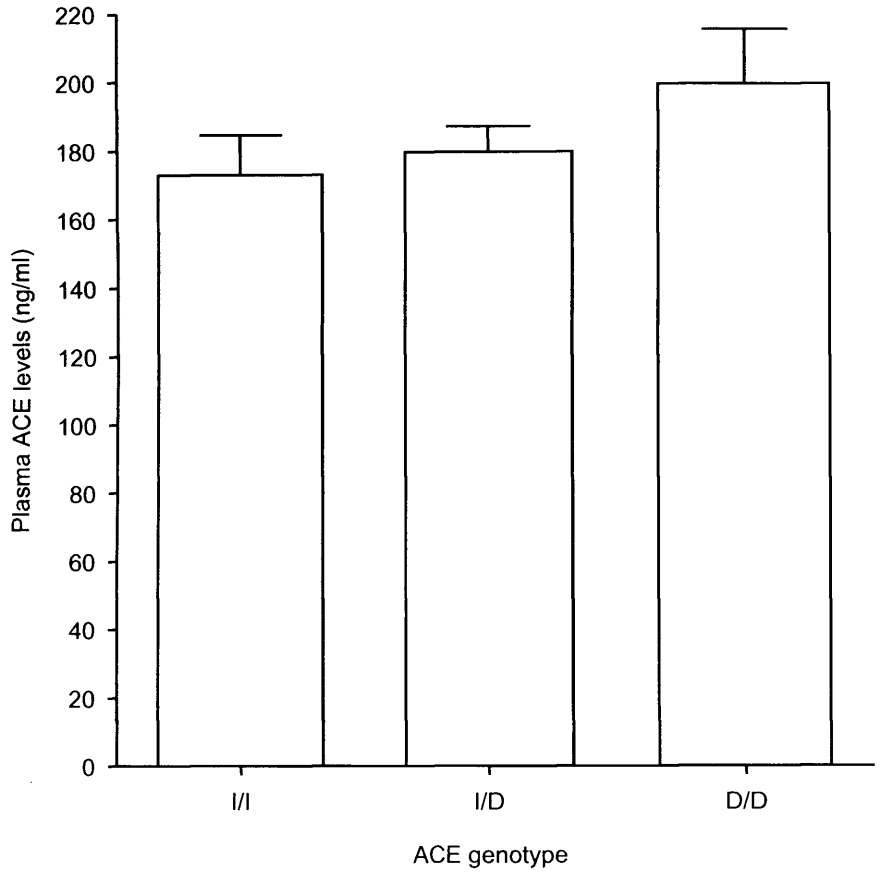


Figure 6.14 Pre-operative plasma ACE levels by genotype

Bars represent mean values, errors are s.e.m. I/I homozygotes appeared to have the lowest levels of protein expression, although this did not reach statistical significance (p=0.298, ANOVA).

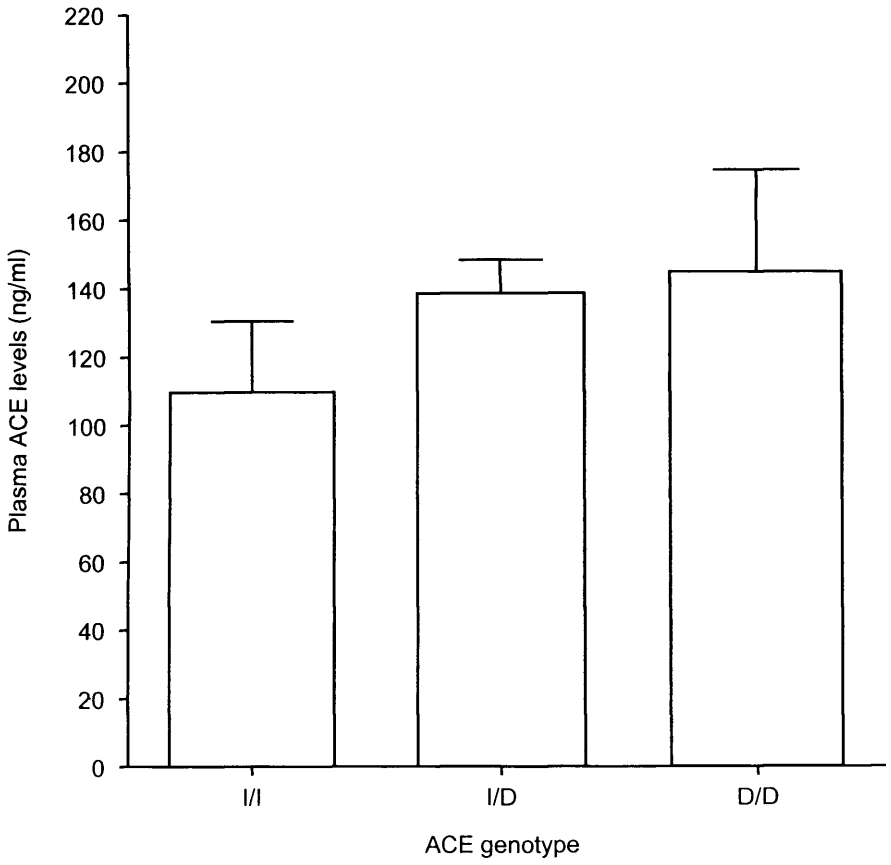


Figure 6.15 Post-operative plasma ACE levels by genotype

Bars represent mean values, errors are s.e.m. I/I homozygotes again appeared to have the lowest levels of protein expression, although this did not reach statistical significance (p=0.420, ANOVA).

Looking at the response to surgery, there was a definite difference in plasma concentration of ACE at both 4hr and 24hr post-operative timepoints, compared to the baseline pre-operative state (183±6 *vs* 132±11 *vs* 133±10 ng/ml, pre-op *vs* 4hr *vs* 24hr timepoints, $p<0.001$, ANOVA, Figure 6.16); post-hoc analysis demonstrated that ACE levels actually fell significantly 4hrs ($p=0.007$, Bonferroni's) and 24hrs ($p<0.001$, Bonferroni's) following surgery. The scale of this fall was not dependent on ACE genotype ($p=0.707$, ANOVA).

Interestingly, post-operatively, there was a concurrent fall in plasma haematocrit, in parallel with the falls in plasma ACE concentration (0.375±0.008 *vs* 0.307±0.008, pre- *vs* 24hrs post-op, $p<0.001$, paired t-test, Figure 6.17). So, the fluid shifts inherent following the stress of surgery and the haemodilution produced by intravenous fluid therapy can be taken

into account by using a previously described formula (Taylor *et al.,* 1976):

$$\text{Corrected cytokine concentration} = \frac{(\text{Cytokine concentration} \times \text{Pre-op-HCT})}{\text{Post-op-HCT}}$$

Interestingly, even when making this adjustment for haemodilution, there remained a trend towards a fall in plasma ACE levels 24hrs following surgery (182±6 *vs* 163±13 ng/ml, pre- *vs* 24hrs post-op, $p=0.097$, paired t-test, Figure 6.18).

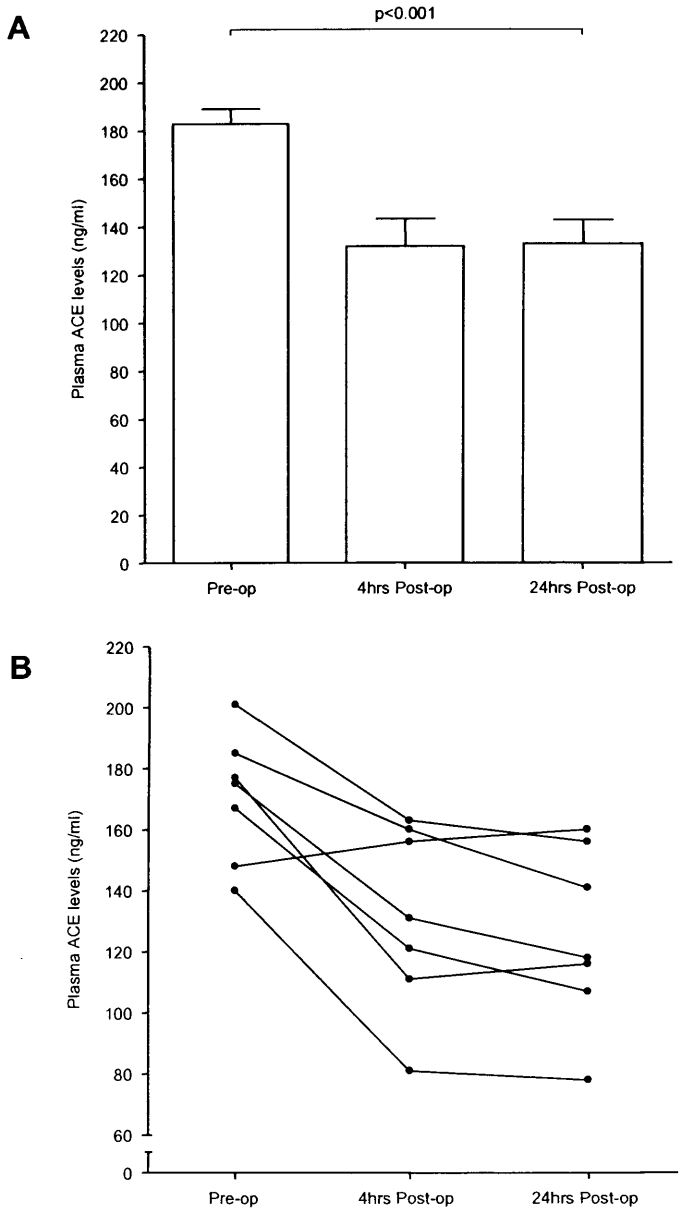


Figure 6.16 Peri-operative plasma ACE levels

A, Bars represent mean values, errors are s.e.m. There was a definite peri-operative fall in ACE levels ($p<0.001$, ANOVA). **B**, Line plot representing results for individual patients with samples available at all 3 peri-operative time points. Each value represents overall grouped mean value, derived from the mean of two separate ELISA experiments.

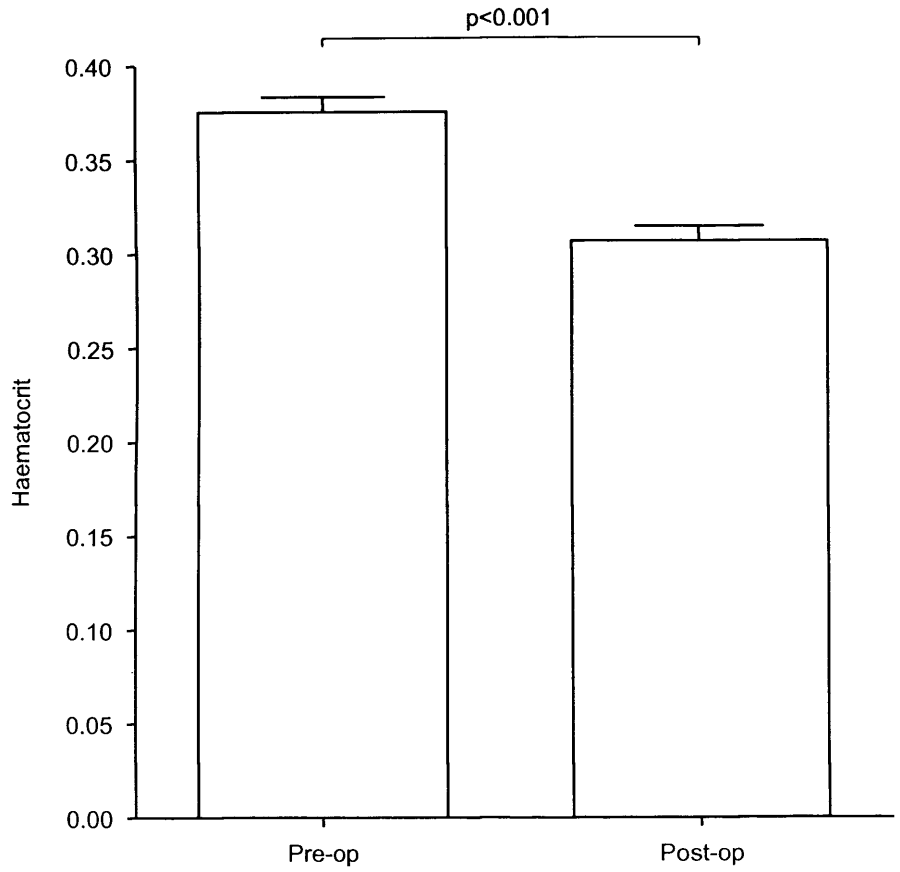


Figure 6.17 Peri-operative haematocrit

Bars represent mean values, errors are s.e.m. Haematocrit fell significantly at the 24hr post-operative timepoint ($p<0.001$, paired t-test).

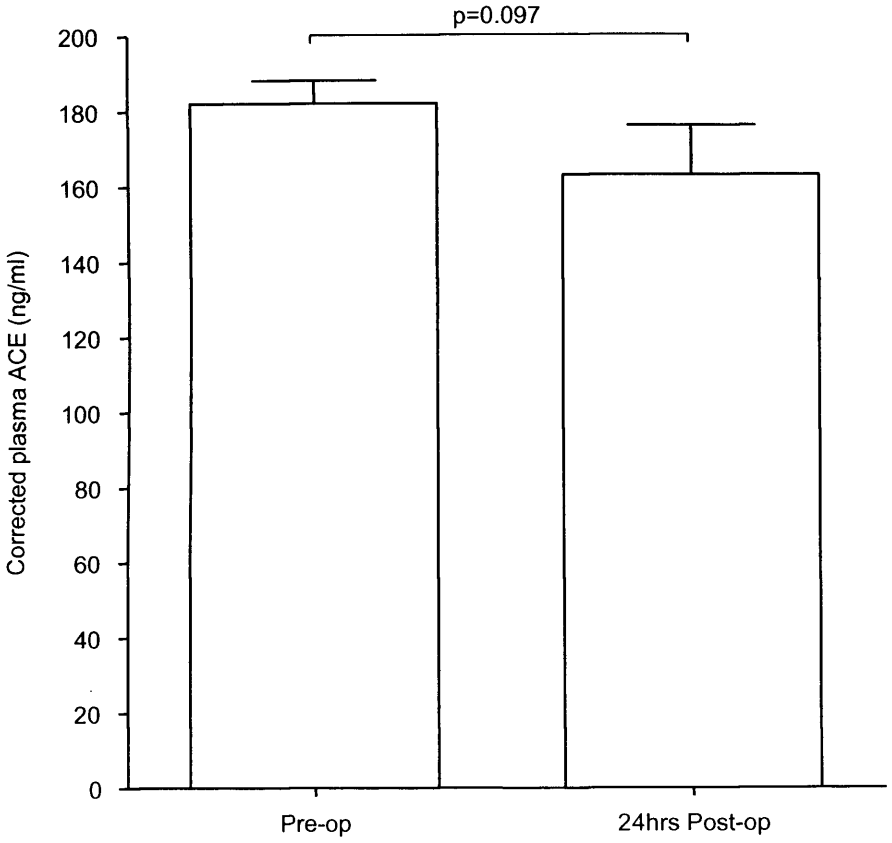


Figure 6.18 Peri-operative plasma ACE levels (corrected for haemodilution)

Bars represent mean values, errors are s.e.m. When plasma concentration results were corrected for haemodilution, there was still a trend towards a fall in levels post-operatively (p=0.097, paired t-test).

Long term ACE expression following surgery

Finally looking at the long term influence of surgery, 10 and 20 weeks following operation, ACE transcription was not restored to pre-operative levels (0.238±0.035 *vs* 0.209±0.027 *vs* 0.341±0.041 IOD, pre-op *vs* 10 weeks and *vs* 20 weeks post-op, p=0.047, ANOVA, Figure 6.19). Post-hoc analysis found that at 20 weeks, transcription was significantly elevated (p=0.043, Bonferroni's). However the small number of available samples made any further sub-analysis unreasonable.

This long term elevation of transcription was mirrored by plasma ACE levels; again protein concentration was altered 10 and 20 weeks after surgery compared to pre-operative baseline (187±9 *vs* 223±6 *vs* 224±9 ng/ml, pre-op *vs* 10 weeks and *vs* 20 weeks post-op, p<0.001, ANOVA, Figure 6.20), with post-hoc analysis confirming significant elevation of concentration at 10 (p=0.001, Bonferroni's) and 20 (p=0.002, Bonferroni's) weeks. As the patients were so far down the line from surgery, it was deemed unnecessary to correct for acute haemodilution.

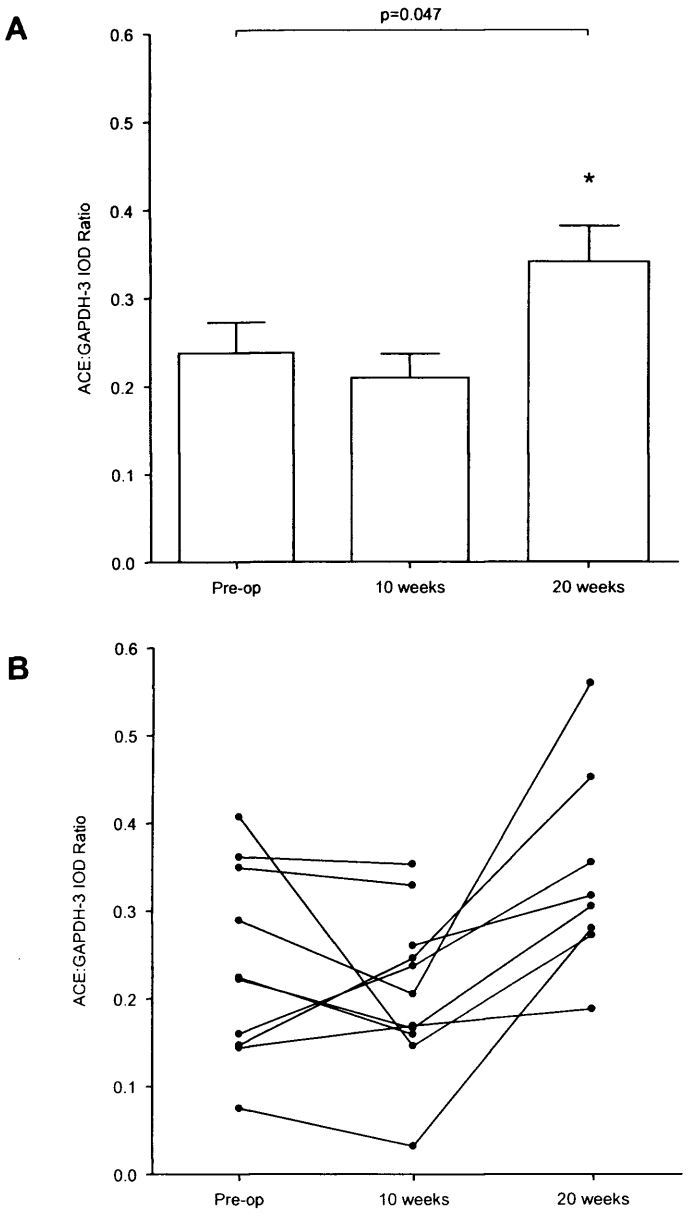


Figure 6.19 Long term ACE gene transcription levels

A, Bars represent mean values, errors are s.e.m. There was a long term rise in transcription post-surgery (p=0.047, ANOVA). * Post-hoc analysis confirmed an upregulation at 20 weeks post-surgery (p=0.043, Bonferonni's). **B**, Line plot representing results for individual patients. IOD, total integrated optical density of ACE:GAPDH-3; each value represents overall grouped mean value, derived from the mean of two separate PCR analyses.

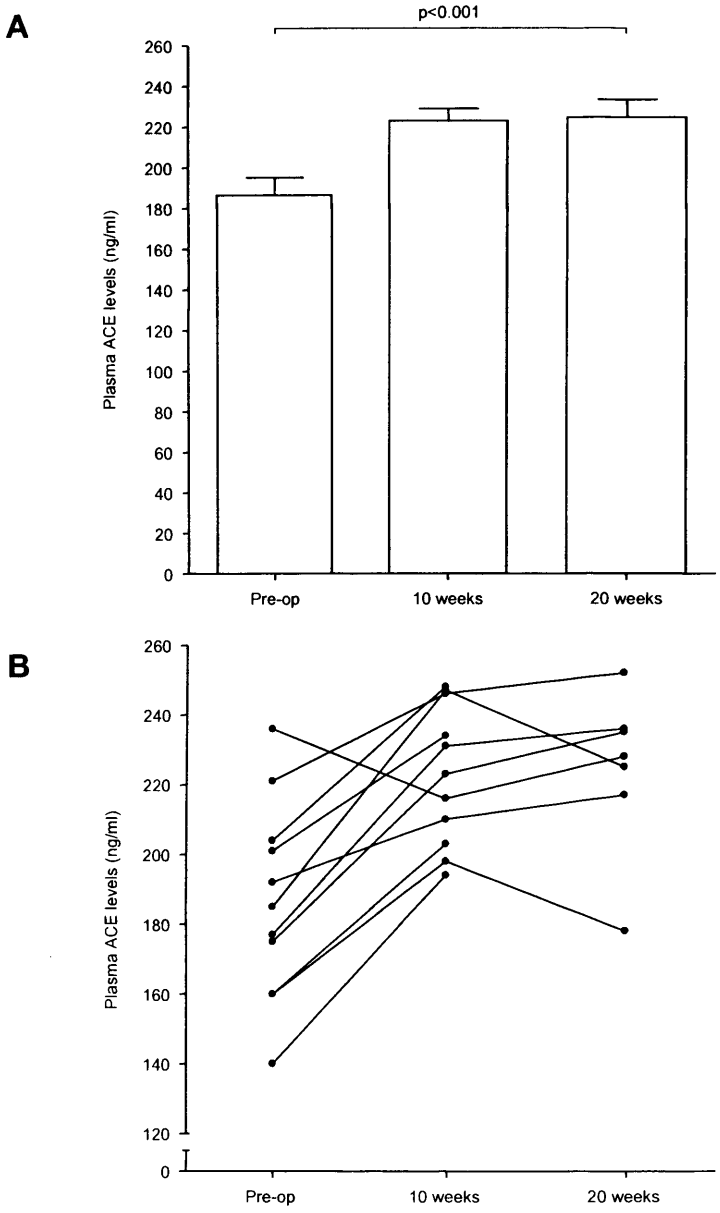


Figure 6.20 Long term plasma ACE levels

A, Bars represent mean values, errors are s.e.m. There was a definite long term post-operative elevation in ACE levels ($p<0.001$, ANOVA). **B**, Line plot representing results for individual patients. Each value represents overall grouped mean value, derived from the mean of two separate ELISA experiments.

6.4 DISCUSSION

The first report of the I/D polymorphism attributed possession of the D-allele with higher protein levels (Rigat *et al.,* 1990). Numerous subsequent gene association studies have confirmed this finding, but these all examined ACE protein levels (see reviews by Woods *et al.,* 2000; Lee *et al.,* 2005). In an attempt to delineate the mechanism underlying this observed association between ACE genotype and circulating protein levels, investigation of ACE gene expression has been conducted. Indeed in the original report of the I/D polymorphism, it was concluded from a family study that genetic control of serum ACE was exerted at the transcriptional level (Rigat *et al.,* 1990).

ACE genotypic influence on transcription has been reported, with higher levels in the circulating peripheral mononuclear cells of D/D homozygotes (Costerousse *et al.,* 1993). Similar results have been reported for tissue, with significantly lower levels of ACE transcription found in I/I homozygote subjects; healthy renal biopsies (Mizuiri *et al.,* 2001) and left ventricular biopsies from ischaemic heart disease patients (Davis *et al.,* 2000) both yielded this same finding. Moreover, ACE activity has also been associated with genotype in a study of post-mortem cardiac tissue samples; D/D homozygotes had the highest levels (Danser *et al.,* 1995).

However, this has not been a uniform finding, with studies of atrial myocardial tissue failing to demonstrate any genotypic influence on ACE gene transcription levels (Tamaki *et al.,* 1997; Spruth *et al.,* 1999), instead suggesting that regulatory control may be exerted by local mechanical forces. Alternatively, the possibility of gene

regulation being cell or tissue specific has been suggested as an explanation for this observed discrepancy (Mizuiri *et al.,* 2001).

No definitive findings were made here, although for the baseline pre-operative state, there was the suggestion of a trend towards lower plasma ACE protein concentration and higher transcription levels with the I/I homozygotes (Figures 6.14 and 6.2). Analysing just the gene transcription results generated from the ACE 2215 primers, reinforced this trend, with I/I homozygotes having significantly higher levels (Figure 6.3). This is in contrast to past reports of ACE gene transcription levels where I/I homozygotes had the lowest levels (Mizuiri *et al.,* 2001). Such discrepancy may be due to tissue-specific differences in RAS behaviour, or the fact that the patients from this study had colorectal cancer. The suggestion of a trend towards lower plasma ACE protein concentrations in I/I homozygotes was maintained at the post-operative timepoint (Figure 6.15).

The overall lack of significant genotypic influence over ACE protein level reported here is unlikely to be due to experimental error, as the intra- and inter-assay co-efficients of variation were within acceptable limits. Instead, it may be due to the relatively small sample size; compounded by the fact that genotype is only responsible for roughly half of the inherent inter-individual variation seen (Rigat *et al.,* 1990).

Nevertheless, this seemingly illogical coupling of elevated plasma levels and reduced gene transcription could be explained by the presence of a negative feedback system of control. Such a mechanism has been described previously for the RAS - increased ACE gene expression and activity have been correlated with increased angiotensin II

production (Muller *et al.,* 1997), and infusion of angiotensin II has produced reduced ACE mRNA levels and activity (Kohara *et al.,* 1992; Schunkert *et al.,* 1993; Metsarinne *et al.,* 1996).

Rather than comparing results from patients grouped by ACE genotype, examination of I/D heterozygotes provided the perfectly controlled environment for studying the individual allelic contribution to transcription. At both the pre- and post-operative timepoints, the D-allele was found to be responsible for a greater proportion of overall gene transcription compared to the I-allele (Figures 6.5 to 6.8). The only previous report to have utilised this methodology for assessing individual allelic contribution to overall ACE transcription, analysed PBMN samples from 12 healthy volunteers (Suehiro *et al.,* 2004). So, an extension of these original findings has been made, with the cohort of colorectal cancer patients recruited for this study.

Combining this breakdown of heterozygote allele contribution, with the transcription results from both I/I and D/D homozygotes, produced an analysis of the individual I- and D-allele contributions within the study population. As expected, the D-allele was still found to be responsible for greater transcription than the I-allele, both pre- and post-operatively (Figures 6.9 and 6.10), again agreeing with the sole past report of allelic breakdown of ACE transcription in the literature (Suehiro *et al.,* 2004). In fact, this grouping of transcription results from homozygotes with the corresponding allele's individual contribution to the heterozygotes' results has not previously been reported; yet it seemingly produces a much more comprehensive reflection of the relative contributions of the I and D alleles to ACE transcription within a population, when compared to the more prevalent method of grouping patients according to the presence

or absence of a particular allele (Lee *et al.,* 2005). Furthermore, this greater D-allele transcription is in line with all the past studies that have reported greater ACE plasma levels and activity to D/D homozygotes (Costerousse *et al.,* 1993; Marshall *et al.,* 2002).

Considering the response to surgery, the mean level of ACE gene transcription was found to be elevated 24 hours following surgery in this study population (Figures 6.11 and 6.12). Given the plethora of evidence pointing towards a role for ACE as a pro-inflammatory cytokine, discussed in Section 1.2, this is an unsurprising finding, as early post-operative inflammation is a key response to injury. Nevertheless, much of the published work to have examined the cytokine response to surgery, has concentrated mainly on TNF-a and interleukins (Lin *et al.,* 2000). Furthermore, this activation of the pro-inflammatory cytokine network has been shown to correlate with both the severity of surgical insult (Sakamoto *et al.,* 1994) and the recovery afterwards (Patel *et al.,* 1994). However, such investigation of the ACE response to surgery has yet to be undertaken.

The existing work involving ACE and surgery has focused on the genotypic influence over outcome, without measuring the actual ACE gene response. In particular, renal transplantation has received attention, due to the known role of the RAS in blood pressure modulation, and the implication of hypertension in graft loss (Abdi *et al.,* 2001; Viklicky *et al.,* 2001), but these studies were only associative. In fact, the only study of ACE within the context of general surgery (oesophagectomy) in the literature (Lee *et al.,* 2005), also concentrated on the association between I- and D-alleles, baseline pre-operative ACE protein levels and the incidence of pulmonary

complications, without examining the actual ACE expression (neither gene nor protein) response to surgery.

When looking at the post-operative ACE gene transcription response to surgery, using the combination of both ACE 216 and ACE 2215 primers, as measured by percentage or absolute changes, there was no association between the I/D genotype and the magnitude of the change. However, when considering just the gene transcription results from the ACE 2215 primers, a definite genotypic pattern emerged, with D/D patients launching a significantly greater response to surgery (Figure 6.12). This could explain the finding of the ACE genotypic influence on gene transcription levels seen pre-operatively, disappearing after surgery. On the other hand, there was no evidence of genotypic association with the ACE plasma protein response to surgery.

Such investigation of the effect of the I/D genotype on peri-operative ACE gene transcription and protein expression has not been previously been reported, but the positive findings are in line with the I/D genotype dependent response to physical training that has been reported in skeletal and left ventricular muscle (Montgomery *et al.,* 1997; Montgomery *et al.,* 1998). Similarly, the adoption of an upright posture has also been found to produce a rise in ACE levels, the scale of which did seem to be genotype-dependent (Woods *et al.,* 2004). However, it is at odds with one past study which examined the ACE response to exercise; serum ACE levels were found to rise after a bout of exercise, but in a non-genotype dependent manner (Woods *et al.,* 2004). Moreover, amongst the work that has found an association between ACE genotype and response to a stimulus, contrary specific relationships have been reported; D/D homozygotes produced the greatest LV muscle increase (Montgomery *et al.,* 1997) but

I/I homozygotes produced a greater anabolic response (Montgomery *et al.,* 1998) following physical training. There has been no previous investigation of the I/D genotypic association with ACE response to an inflammatory stimulus, so the finding that D/D homozygotes produced the largest response to surgery is seemingly novel.

It is unclear why there should have been evidence of a genotypic association with the gene transcription response, but none with the change in plasma protein levels. It may be that any ACE genotype-dependent effects are mediated at the tissue level, making systemic measures of plasma ACE of secondary importance. Alternatively, the lack of association with the plasma protein response may have been due to it being controlled by a number of other genetic and environmental factors, which have not been taken into account for. Similarly, the great magnitude and uneven nature of the surgical insult applied to this study cohort of patients may have prevented the detection of any genotype-association. It is equally uncertain why the significant genotypic association only emerged when one set (ACE 2215) of primers alone were considered. This may have been due to differences in base pair composition and structure of the target sequence, primer efficiency, expected product length or the experimental error involved in performing experiments on different days with different ambient temperatures.

Rather than looking at genotypes, in I/D heterozygotes within this study population, there also seemed to be an allelic difference in the response to surgery. Although there was no significant difference peri-operatively in the absolute degree of allele transcription, a different picture emerged when the relative contributions were analysed. The degree of dominance of the D-allele in contributing to overall ACE gene transcription was lessened significantly following surgery, perhaps suggesting that in

I/D heterozygotes, the I-allele mounted a relatively greater response (Figure 6.13). It may be that one cannot extrapolate this finding to homozygotes, as the corollary would be a greater response by I/I compared to D/D subjects, and the opposite was observed (Figure 6.12). Nevertheless, the suggestion of a greater I-allele reaction to the stimulus of surgery is in line with the superior response to physical training demonstrated previously by I/I homozygotes (Montgomery *et al.,* 1998; Montgomery *et al.,* 1999). Whether such an allelic difference in response may actually be of relevance, in terms of determining clinical outcome will be analysed and discussed later in Chapter 7.

Thus, the finding of an upregulation in ACE gene transcription fits with the scheme of general pro-inflammatory cytokine stimulation, yet has hitherto not been reported. Similarly, the predominance of the D-allele transcription both pre- and post-operatively in colorectal cancer patients is novel, as is the evidence of a relatively greater peri-operative response to surgery in D/D homozygotes. Caution is obviously required with the small number of patients within this study, and a larger cohort would be needed before more conclusive statements could be made. Nevertheless these remain interesting findings.

However, and surprisingly, the peri-operative plasma ACE protein response did not mirror gene transcription, as might initially be expected. Plasma ACE levels were instead found to be decreased at both 4 and 24 hours following surgery (Figure 6.16). Although ACE activity, rather than plasma protein concentration, was the measure, such a post-operative fall has been reported following thoracic surgery (Karnezis, 1999). Similarly, following the inflammation-provoking cardiopulmonary bypass, ACE activity has also been found to drop (Gorin *et al.,* 1986). Conversely, isolated

pulmonary (rather than systemic) ACE activity has been reported to rise in the peri-operative period around total knee arthroplasty surgery (Jules-Elysee *et al.,* 2004).

The observed post-operative fall in ACE plasma levels is novel, and may potentially be due to consumption of the protein at the operative site by the inflammatory response. Such a hypothesis has been suggested as the reason for the observed fall in IL6 soluble receptor protein following colorectal cancer surgery (Hatada *et al.,* 2000). The existence of a lag-time in protein production following the inflammatory stimulus of surgery (Panelli *et al.,* 2002) may underlie the discrepancy between gene transcription and plasma levels. So, the 24 hour post-operative sampling timepoint may have been too early to detect a positive association between transcription and protein production, and such a relationship may have only emerged later. Alternatively, the reciprocal relationship between transcription and protein levels may reflect a negative feedback mechanism of expression control; the immediate fall in plasma ACE concentration following the inflammatory stimulus of surgery may stimulate an increase in corresponding gene transcription, as a compensatory measure to restore protein levels. The experimental evidence supporting such a possibility has been outlined, with infusion of Ang II shown to result in reduced ACE mRNA levels (Metsarinne *et al.,* 1996).

Interestingly, even when account was taken for the inevitable fluid shifts provoked by surgery and intravenous fluid therapy, by using the haematocrit as a corrective factor, there was still a trend towards a fall in ACE concentration (Figure 6.18). Although not in widespread use, such a methodology has been previously employed during the investigation of the cytokine response to CABG surgery (Roth-Isigkeit *et al.,* 1999);

peri-operative haemodilution was found to exert significant influence over plasma cytokine measurements, and a corrective factor based on haematocrit was championed. The findings from this study were in line with this. Furthermore, the fact that ACE activity, rather than just concentration, has also previously been found to fall post-operatively (Karnezis, 1999; Gorin *et al.,* 1986), suggests that the observed post-operative fall in ACE plasma concentration may represent a real effect rather than a corollary of haemodilution.

Finally, the long term post-operative ACE response was analysed; gene transcription was raised at 20 weeks (Figure 6.19), and plasma protein levels were also elevated at both 10 and 20 weeks following surgery (Figure 6.20). This prolonged activation of the RAS at an intermediate timepoint following surgery has not previously been reported. However, one study which followed patients for one year after CABG surgery, found plasma ACE activity to be elevated compared to pre-operative baseline levels in subjects possessing the D-allele (Van Geel *et al.,* 2003). The significance of such a rise in long-standing plasma ACE concentration is clearly dependent on the stability of levels over time. Several short-term environmental stimuli have been found to influence ACE levels, such as meal ingestion (Jalil *et al.,* 1999), posture and acute exercise (Woods *et al.,* 2004); consequently ACE activity within individuals may differ by more than 100% over a 6 month period (Ribichini *et al.,* 1998). Nevertheless, overall, ACE levels have still been found to retain long-term stability (Beneteau-Burnat *et al.,* 1990), increasing the likelihood that the observed prolonged ACE elevation was a genuine observation.

As to the potential functional implications of this prolonged activation of the RAS, there are a few possibilities. A systemic inflammatory response, as reflected by enhanced levels of pro-inflammatory cytokines, has been associated with hypermetabolism-associated malnutrition (Delano *et al.,* 2006). More pertinently to this study, tumour-related malnutrition has also been associated with a systemic inflammatory response (Falconer *et al.,* 1994; Argiles *et al.,* 2003), so it may be that the prolonged elevation of ACE may be responsible for post-operative malnutrition and weight loss. Specifically, elevated concentrations of inflammatory mediators such as CRP and IL6 have been associated with loss of muscle in the elderly (Schaap *et al.,* 2006). So, it is possible that the similarly pro-inflammatory ACE is also involved in mediating muscle loss. Indeed, in a murine model, a role for Ang-II in mediating muscle atrophy, via a mechanism of protein degradation has been reported (Sanders *et al.,* 2005). The nuclear transcription factor NF-?B has been identified as a possible mediating factor for this response (Russell *et al.,* 2006).

Furthermore, as well as bulk, a systemic inflammatory response has also been associated with poorer function, manifesting as muscle strength and general physical performance (Bautmans *et al.,* 2005; Cesari *et al.,* 2004). Given the documented presence and known functional role of the RAS in skeletal muscle (Woods *et al.,* 2000), it may be that the prolonged post-operative elevation of ACE observed may reflect or mediate the poorer muscle function and performance often evident in patients following surgery. Clearly this is speculation, and further research is required to investigate the significance of the prolonged activation of the RAS.

The main findings of this investigation of the effects of surgery for colorectal cancer on peri-operative and long term ACE, and the influence of the I/D polymorphism are summarised:

- Starting with the baseline pre-operative state, there was evidence of lower plasma ACE protein concentration and higher transcription levels in I/I homozygote colorectal cancer patients (Figures 6.2, 6.3 and 6.14).
- Focusing on the I/D heterozygote patients, there was significant evidence of greater contribution to gene transcription from the D-allele, compared to the I-allele. This was the case both pre-operatively and following surgery (Figures 6.5 to 6.8).
- Expanding this to the whole study population by combining this allelic breakdown with results from homozygote patients demonstrated the D-allele to produce greater transcription than the I-allele (Figures 6.9 and 6.10).
- There was a rise in mean ACE transcription level following surgery, the scale of which was influenced by the I/D polymorphism when considering the results from the ACE 2215 primers in isolation, with D/D homozygotes launching the greatest response (Figures.6.11 and 6.12).
- However, in the I/D heterozygotes, the dominant contribution of the D-allele to transcription was lessened following surgery, suggesting a relatively greater I-allele response to the inflammatory stimulus in these patients (Figure 6.13).
- There was a post-operative fall in plasma ACE protein levels, which remained significant even after correction for the concomitant change in haematocrit (Figures 6.16 to 6.18), and was unassociated with I/D genotype.
- In the long term, both ACE transcription and plasma protein levels were elevated 20 weeks following surgery (Figures 6.19 and 6.20).

So far, the peri-operative and long term responses of the pro-inflammatory IL6 and ACE genes, along with the roles of the -174 G/C and I/D polymorphisms, have been described. However, no analysis of the possible functional effects of this inflammatory reaction to surgery has been made, and this will be addressed in the next chapter, with particular reference to the potential influence on patient outcome.

CHAPTER 7

ACE and IL6 Genotypes, Expression and Outcomes

CHAPTER 7

7.1 INTRODUCTION

Initially prompted by the difficulties in trying to differentiate between the systemic inflammatory response syndrome (SIRS) and sepsis in the early post-operative period, the cytokine response to surgery and its association with outcome has become an increasingly popular topic of study. As aortic aneurysm surgery (and in particular the cross-clamping of the aorta) produces a particularly florid inflammatory response, there has been much investigation of this procedure in an attempt to identify potential prognostic and diagnostic indicators. Thus, patients with a greater pro-inflammatory cytokine (IL1ß and TNFa) response to AAA surgery have been found to be at greater risk of ARDS, MOF and mortality (Roumen *et al.,* 1993). As an integral mediator of the acute phase response to injury, the IL6 response has been of particular interest, and an excessive or prolonged rise in circulating protein levels has been associated with both morbidity and mortality in elective aortic surgery patients (Baigrie *et al.,* 1993). Although this post-operative rise in IL6 concentration has been consistently reported, the association with poorer outcome has not been a uniform finding (Swartbol *et al.,* 2001) However, similar correlation has been made in burns victims (Schluter *et al.,* 1991) and trauma patients (Ertel *et al.,* 1990). Moreover, in the emergency setting of ruptured AAA surgery, higher IL6 protein levels have proved predictive of poorer outcome, as defined by the development of MOF (Bown *et al.,* 2004).

Similarly, in major abdominal surgery, raised post-operative IL6 protein levels have been associated with septic morbidity (Mokart *et al.,* 2002), especially in patients with a clinical assessment of SIRS (Mokart *et al.,* 2005). However, no correlation with the development of SIRS following the more routine colorectal surgery has been identified

(Sarbinowski *et al.,* 2005). Moreover, despite its increasingly evident pro-inflammatory role, no investigation of the influence of the ACE response to any type of surgery on clinical outcome has been conducted.

Genetic variants influence an individual's susceptibility to disease (Lohmueller *et al.,* 2003). Furthermore, the outcome from surgery is shaped by a combination of a patient's ability to tolerate the procedure, and their response to it, both in turn also influenced by genotype. To date, the investigation of patient fitness has formed the basis of much of pre-operative assessment, attempting to discern the ability to tolerate surgery. However, the discovery of a number of functional polymorphisms in genes encoding cytokine proteins that co-ordinate the acute inflammatory response has driven increasing interest in the patient genetic-based response to surgery (Lee *et al.,* 2006). Thus, given the fact that racial groups differ in the pattern of genetic polymorphisms found within their populations (Hoffman *et al.,* 2002), it may be that such inherited differences underlie the ethnic differences in surgical outcome described in Section 1.5.

More specifically, the functional -174 G/C polymorphism of the key pro-inflammatory cytokine IL6, first described in juvenile chronic arthritis patients (Fishman *et al.,* 1998), has been investigated in surgical patients. Although in renal transplant patients, its significance remains unclear (Muller-Steinhardt *et al.,* 2002; Hutchings *et al.,* 2002), in those undergoing CABG, GG homozygotes have been found to be at greater risk of developing post-operative complications (Gaudino *et al.,* 2003) and a longer stay in hospital (Burzotta *et al.,* 2001). Similarly, possession of the G-allele has been associated with development of post-operative organ failure following major AAA surgery (Bown *et al.,* 2004). However, no such investigation has been conducted in

abdominal surgery, and the possible prognostic significance of the -174 G/C polymorphism remains is yet to be determined in this setting.

On the other hand, the I/D polymorphism of the ACE gene, which is being increasingly thought of as multifunctional (as described in Section 1.2), has received much less attention. Again, the investigation of outcome in renal transplant patients has proved inconclusive thus far (Abdi *et al.,* 2001; Viklicky *et al.,* 2001). Otherwise, there has only been one other reported study investigating the I/D polymorphism and surgical outcome, and this concluded that oesophagectomy patients possessing the D-allele were at greater risk of developing post-operative pulmonary complications (Lee *et al.,* 2005). Thus, the investigation of this ACE polymorphism in influencing post-operative outcome in colorectal cancer patients again remains to be seen.

7.2 MATERIALS AND METHODS

As described in Chapter 3, following ethical approval and informed consent, blood samples were taken from elective colorectal cancer surgery patients pre-operatively and, when possible, 4 hours and 24 hours following surgery. PBMNs and plasma were separated from blood by a process of centrifugation through a density gradient polymer and PBMN nucleic acids extracted using a phenol-ethanol precipitation method (see Sections 3.2 and 3.3).

ACE and IL6 genotype were established from PBMN-isolated DNA using PCR, with products being separated by gel electrophoresis (see Section 3.4). From PBMN-isolated total cellular RNA, a semi-quantitative assessment of levels of gene transcription for both IL6 and ACE was made, using specific primers and an RT-PCR reaction, with

normalisation to the house-keeping gene GAPDH-3 (see Sections 5.2 and 6.2 and Chapter 3).

Levels of plasma protein for both IL6 and ACE were determined using a commercially available ELISA kit (R&D Systems, UK), with samples run in duplicate, and the final result representing the mean of two separate experimental plates (see Sections 5.2 and 6.2 and Chapter 3).

A prospective record of post-operative complications and length of stay was made, taken from the patient notes.

7.3 RESULTS

The patient demographics, operative details and genotypes correspond to those already described in Chapter 4. Initial analysis with the Kolmogorov-Smirnov test demonstrated the LOS data (see Appendix II) and IL6 data (both gene transcription and protein expression; Appendices IV and V) to not be normally distributed, prompting the presentation of data as median (range) and the use of non-parametric statistical tests when making group comparisons. However, ACE data (both gene transcription and protein expression; Appendices IV and V) did prove to be normally distributed using the Kolmogorov-Smirnov test, leading to the presentation of data as mean ± s.e.m. and the use of parametric statistical tests when making group comparisons.

Outcome as determined by LOS

Starting with LOS as the surgical outcome measure, as discussed in Section 4.4, no association between LOS and the -174 G/C IL6 genotype was evident (12.5 (9-15) *vs* 11 (8-25) *vs* 15 (8-31) days, G/G *vs* G/C *vs* C/C genotypes, p=0.718, Kruskal-Wallis, Figure 7.1). Even if patients were grouped according to the presence (C/C and G/C genotypes) or absence (G/G homozygotes only) of a C-allele, there was no difference in LOS (11 (8-25) *vs* 15 (8-31) days, C-allele present *vs* G/G genotypes, p=0.416, Mann-Whitney U). Similarly, if patients were divided into "short-stayers" and "long-stayers" depending on whether they had LOS =10 days or not (see Section 4.4), there were still very similar IL6 genotype frequencies between the "short-stayers" and "long-stayers" (p=0.946, Chi^2, Figure 7.2).

In the same way, there appeared to be no association between ACE I/D genotype and LOS (10 (8-17) *vs* 14 (9-31) *vs* 13 (8-25) days, I/I *vs* I/D *vs* D/D genotypes, p=0.233, Kruskal-Wallis, Figure 7.3). However, if patients were grouped according to the presence (D/D and I/D) or absence (I/I) of a D-allele, then a trend was observed towards a longer post-operative stay in individuals carrying a D-allele (13.5 (8-31) *vs* 10 (8-17) days, D-allele present *vs* I/I homozygotes, p=0.097, Mann-Whitney U, Figure 7.4). Similarly, if patients were divided into "short-stayers" and "long-stayers" depending on whether they had LOS =10 days or not (see Section 4.4), then differences in ACE genotype frequencies between the two groups emerged.

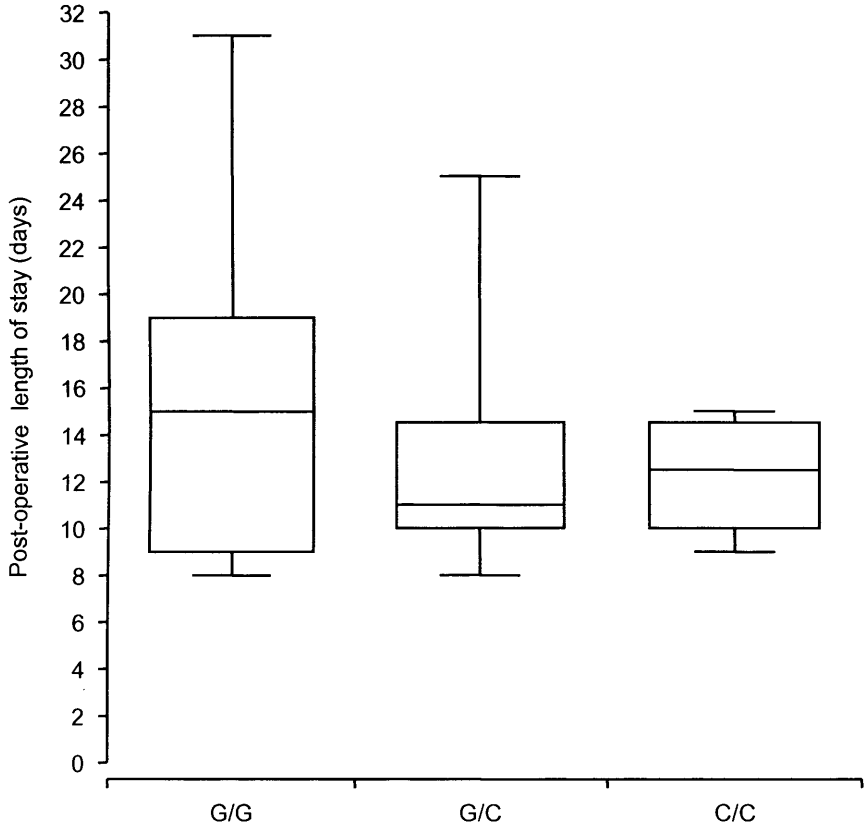


Figure 7.1 LOS by IL6 -174 G/C genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was no significant difference in LOS between the three IL6 genotype groups (p=0.718, Kruskal-Wallis).

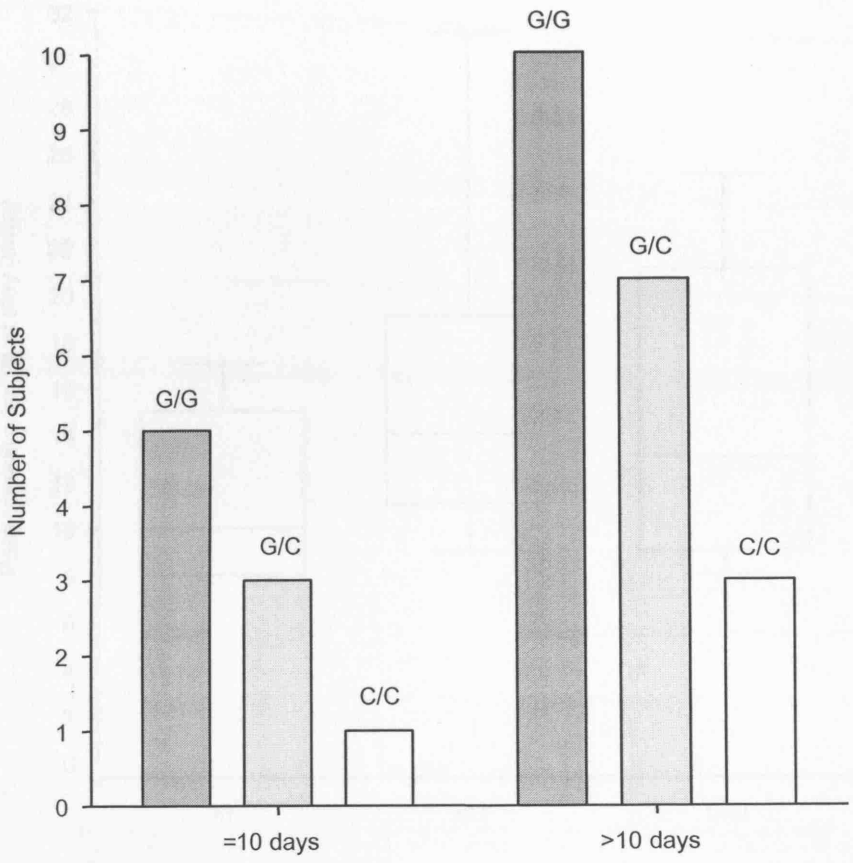


Figure 7.2 LOS breakdown by IL6 -174 G/C genotype

Bars represent numbers of subjects. There was an equal distribution of IL6 genotype frequencies in both short- and long-stayers ($p=0.946$, Chi^2).

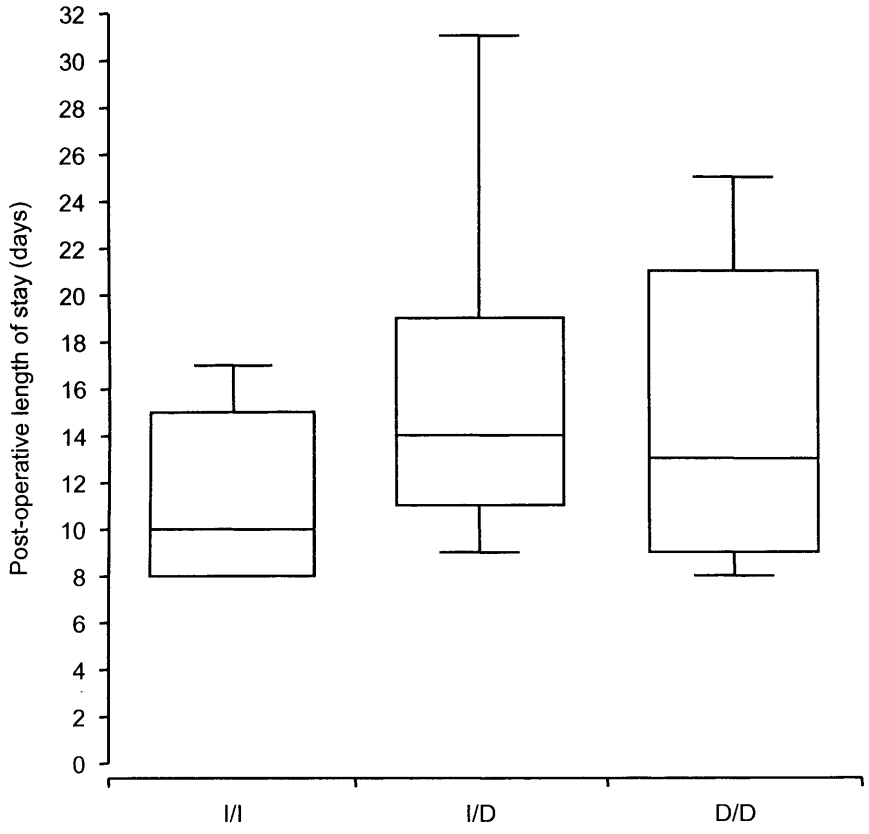


Figure 7.3 LOS by ACE I/D genotype

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was no significant difference in LOS between the three ACE genotype groups (p=0.233, Kruskal-Wallis).

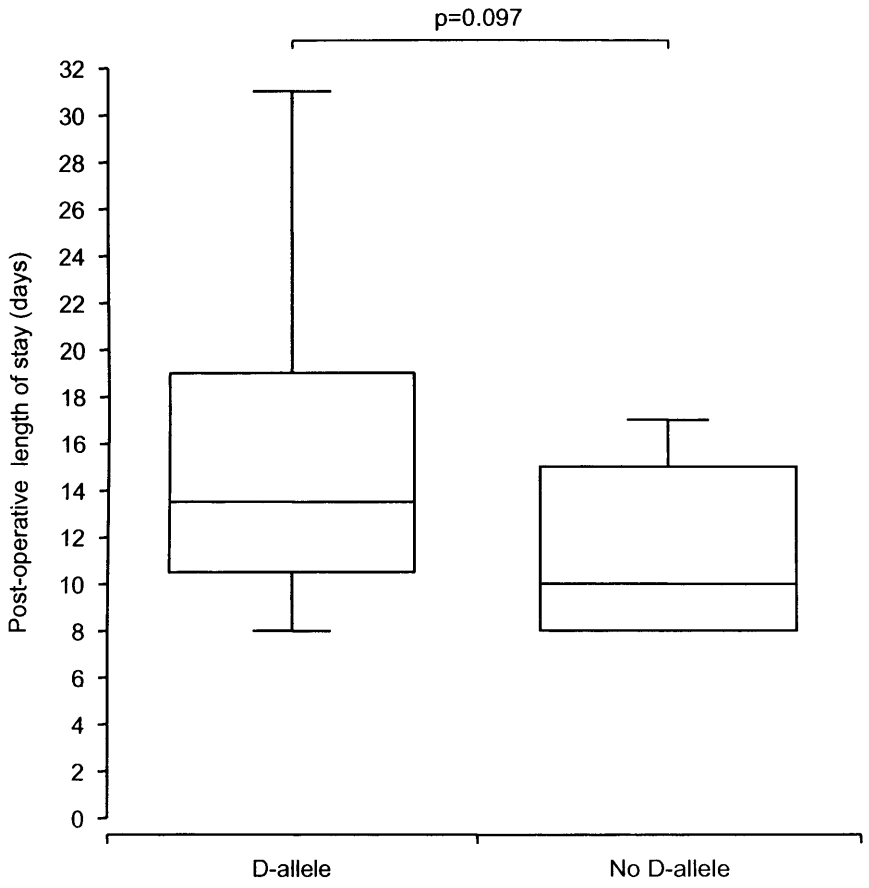


Figure 7.4 LOS by presence of ACE D-allele

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a trend towards those patients possessing a D-allele having a longer post-operative LOS (p=0.097, Mann-Whitney U).

There was a trend for a pre-ponderance of I/I homozygotes with a LOS =10 days, when measured against D-allele carriers (p=0.087, Chi^2, Figure 7.5). Indeed on further analysis, compared to D-allele carriers, these I/I homozygotes had an Odds Ratio of 4.5 (0.8-27.4, 95% C.I.) of having a LOS =10 days.

Analysis of the influence of the gene response to surgery was also assessed by dividing the patients into "short-stayers" and "long-stayers" groups as above. Starting with IL6 gene transcription, despite there being no difference between "short" and "long-stayers" (0.116 (0.033-0.240) *vs* 0.125 (0.057-0.357) IOD, "short" *vs* "long", p=0.710, Mann-Whitney U), there was a suggestion of a positive correlation between pre-operative levels of IL6 gene transcription and LOS (p=0.122, R=0.305, Spearman's rank correlation, Figure 7.6A). Interestingly, pre-operative IL6 gene transcription significantly correlated with pre-operative CRP levels (p=0.009, R=0.502, Spearman's rank correlation, Figure 7.6B). However, neither post-operative transcription levels (0.275 (0.062-0.454) *vs* 0.174 (0.053-0.699) IOD, "short" *vs* "long", p=0.248, Mann-Whitney U), nor the gene response to surgery, calculated as a percentage change, (20 (-40-388) *vs* 50 (-56-371) %, p=0.760, Mann-Whitney U) differed between these two groups.

Looking to see if these effects on IL6 gene transcription were mirrored by those for circulating protein levels, both 24 hour post-operative IL6 levels (120 (6-123) *vs* 105 (5-321) ng/ml, "short" *vs* "long", p=0.772, Mann-Whitney U), and the surgery-induced change in concentration (103 (6-123) *vs* 105 (-13-321) ng/ml, p=0.591, Mann-Whitney U) again were not associated with LOS.

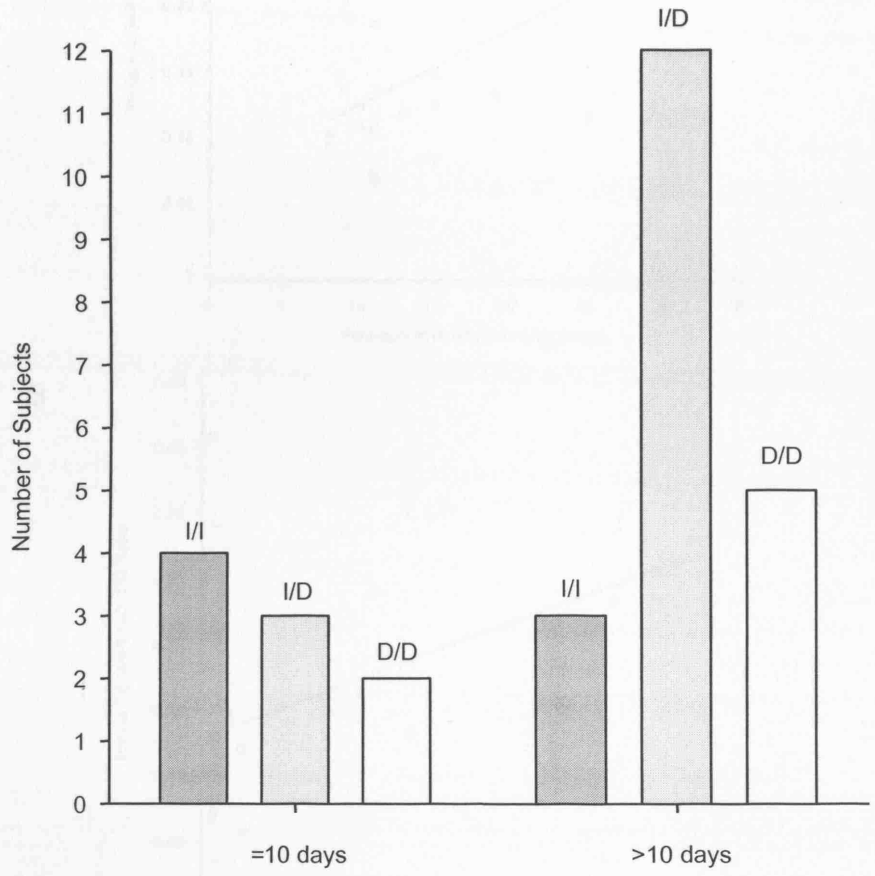


Figure 7.5 LOS breakdown by ACE I/D genotype

Bars represent numbers of subjects. There was seemingly a pre-ponderance of I/I homozygotes in the group who had LOS =10 days compared to the longer-stayers ($p=0.087$, Chi^2).

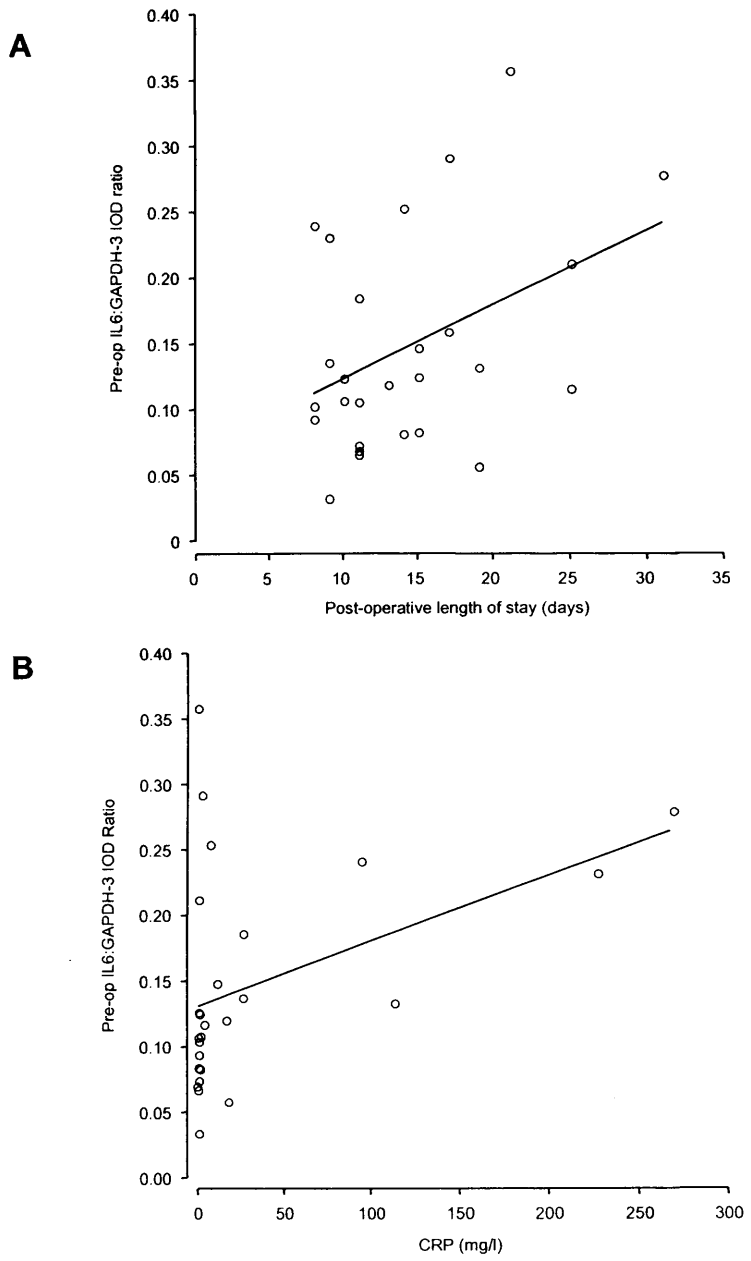


Figure 7.6 LOS against pre-operative IL6 gene transcription and CRP

Points represent individual patient cases. IOD, integrated optical density. CRP, C-reactive protein. **A**, There seemed to be a positive correlation between LOS and pre-operative IL6 gene transcription (p=0.122, R=0.305, Spearman's rank correlation). **B**, There was a positive correlation between pre-operative CRP and IL6 gene transcription (p=0.009, R=0.502, Spearman's rank correlation).

There was no difference in pre-operative baseline (0.220±0.046 *vs* 0.272±0.026 IOD, p=0.344, unpaired t-test) and 24 hour post-operative (0.378±0.047 *vs* 0.318±0.035 IOD, p=0.339, unpaired t-test) ACE gene transcription between those patients who had LOS =10 days and the remainder with LOS >10 days. However, the situation was different when looking at the ACE gene transcription response to surgery. Those with a LOS =10 days had a definite tendency towards a greater percentage change in ACE transcription than the "long-stayers" (106 (-14-148) *vs* -11 (-49-136) %, "short-" *vs* "long-stayers", p=0.061, Mann-Whitney U, Figure 7.7). Even without this grouping, there was a definite negative correlation between percentage change in ACE gene transcription and LOS (p=0.055, R=-0.436, Spearman's rank correlation, Figure 7.8).

As for the influence of circulating protein levels, there was also no difference in baseline (194±9 *vs* 178±8 ng/ml, "short-" *vs* "long-stayers", p=0.221, unpaired t-test) and 24 hour post-operative (142±11 *vs* 130±13 ng/ml, "short-" *vs* "long-stayers", p=0.512, unpaired t-test) ACE concentrations between the "short-stay" and "long-stay" groups. On the other hand, unlike ACE gene transcription, the post-operative change in ACE protein levels also seemed to be unaffected (-35±9 *vs* -47±7 ng/ml, "short-" *vs* "long-stayers", p=0.339, unpaired t-test).

Outcome as determined by the occurrence of post-operative complications

As discussed previously in Section 4.3, there was no mortality in the study population. Moving on to the other useable measure of surgical outcome, three patients developed post-operative complications, and their cases have already been described in Section 4.3.

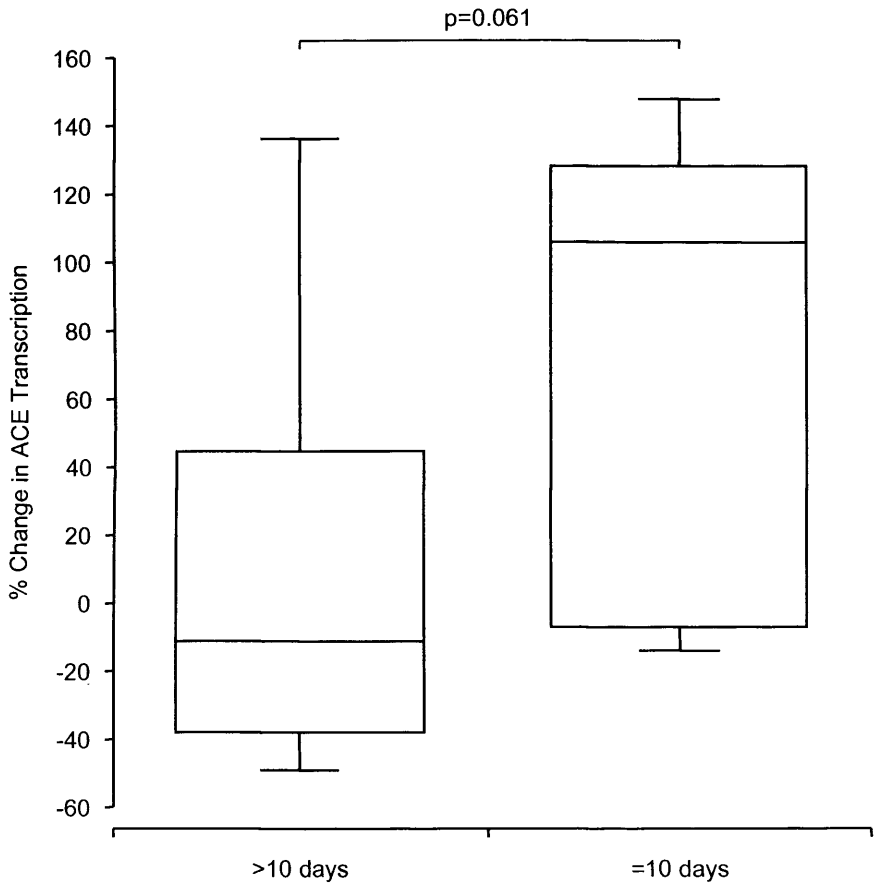


Figure 7.7 LOS breakdown by post-operative change in ACE gene transcription

Box and whisker plots representing median (line) values with quartiles (box) and range (error). There was a definite tendency for patients with LOS =10 days to have launched a greater ACE gene transcription response to surgery (p=0.061, Mann-Whitney U).

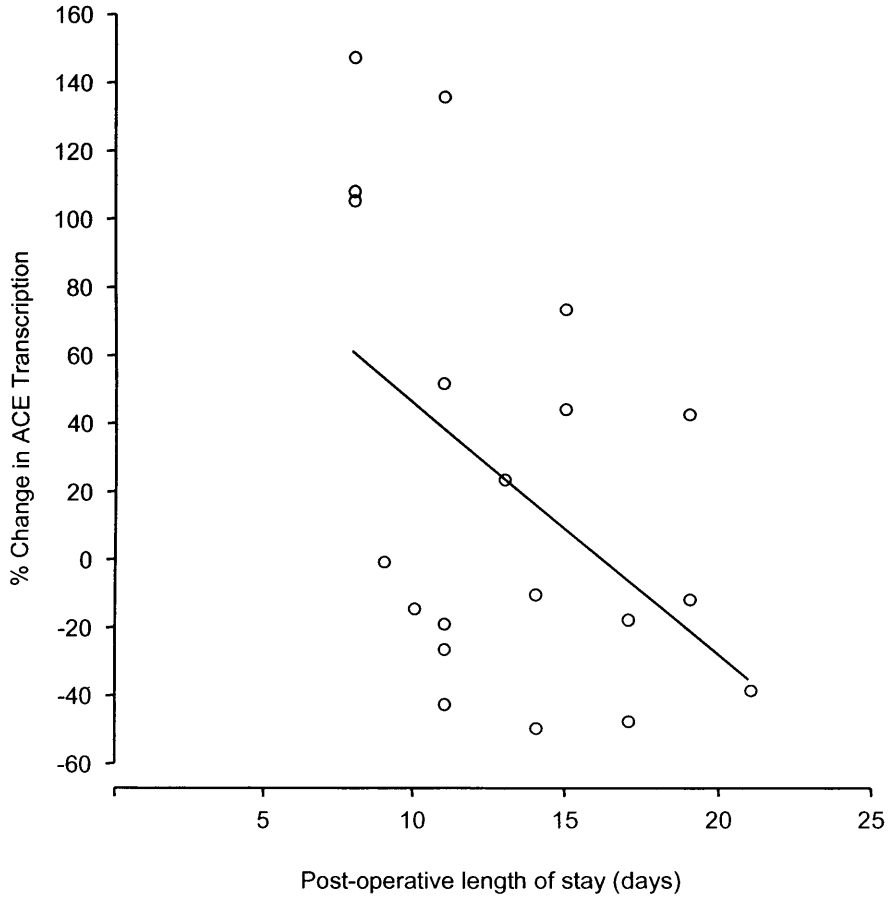


Figure 7.8 LOS against post-operative change in ACE gene transcription

Points represent individual patient cases. There was a negative correlation between LOS and the percentage change in ACE gene transcription following surgery ($p=0.055$, $R=-0.436$, Spearman's rank correlation).

Although the small number in this group limits somewhat meaningful analysis, it is interesting to note that there was no difference in LOS between those who did and did not experience post-operative complications (15 (11-21) *vs* 11 (8-31) days, complications *vs* no complications, p=0.330, Mann-Whitney U).

The development of post-operative complications seemed to be unaffected by IL6 genotype (12G/G's - 10G/C's - 4C/C's *vs* 3G/G's - 0G/C's - 0C/C's, no complications *vs* complications, p=0.210, Chi^2). However, all three such patients were G/G homozygotes and a definite trend was evident towards those with a C-allele having a better outcome (14 *vs* 0 C-allele carriers, no complications *vs* complications, p=0.077, Chi^2, Figure 7.9). Despite there being also no association between ACE genotype (7I/I's - 13I/D's - 6D/D's *vs* 0I/I's - 2I/D's - 1D/D's, no complications *vs* complications, p=0.586, Chi^2) or D-allele possession (19 *vs* 3 D-allele carriers, no complications *vs* complications, p=0.302, Chi^2), and post-operative complications, interestingly none of these patients were ACE I/I homozygotes.

Moving from genotype to gene function, neither pre-operative (0.185 (0.083-0.357) *vs* 0.122 (0.033-0.291) IOD, complications *vs* no complications, p=0.355, Mann-Whitney U), post-operative levels of IL6 gene transcription (0.231 (0.157-0.699) *vs* 0.194 (0.053-0.620) IOD, p=0.315, Mann-Whitney U), absolute gene transcription response to surgery (0.148 (-0.201-0.514) *vs* 0.046 (-0.093-0.488) IOD, p=0.634, Mann-Whitney U), nor percentage change in gene transcription (178 (-56-277) *vs* 37 (-42-388) %, p=0.634, Mann-Whitney U) seemed to differ in those who developed complications.

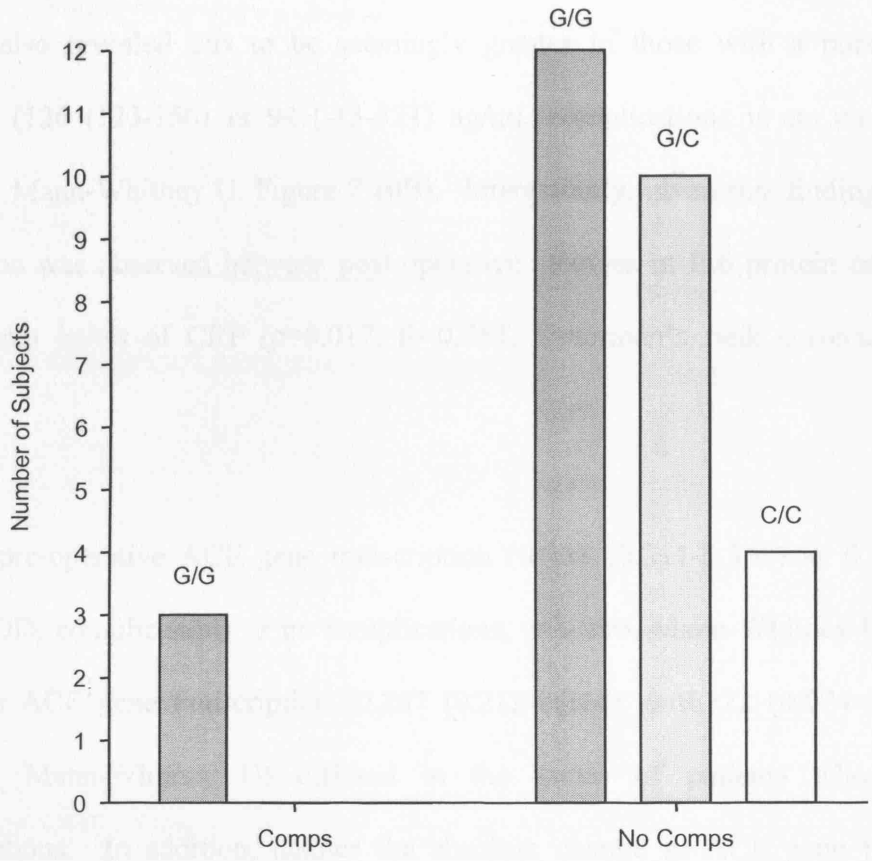


Figure 7.9 Complications breakdown by IL6 -174 G/C genotype

Bars represent numbers of subjects. Comps, complications. There was a trend towards C-allele carriers (C/C and G/C patients) having a better outcome ($p=0.077$, Chi^2).

However, a trend was observed towards patients who developed complications having greater post-operative levels of IL6 protein, 24 hours following surgery (126 (123-152) *vs* 94 (5-321) ng/ml, complications *vs* no complications, p=0.078, Mann-Whitney U, Figure 7.10A). Analysis of the change in IL6 protein concentration provoked by surgery also revealed this to be seemingly greater in those with a poorer surgical outcome (126 (123-150) *vs* 94 (-13-321) ng/ml, complications *vs* no complications, p=0.078, Mann-Whitney U, Figure 7.10B). Interestingly, given this finding, a positive correlation was observed between post-operative changes in IL6 protein concentration and plasma levels of CRP (p=0.017, R=0.553, Spearman's rank correlation, Figure 7.11).

Neither pre-operative ACE gene transcription (0.343 (0.211-0.360) *vs* 0.240 (0.042-0.497) IOD, complications *vs* no complications, p=0.396, Mann-Whitney U) nor post-operative ACE gene transcription (0.267 (0.212-0.314) *vs* 0.322 (0.074-0.555) IOD, p=0.159, Mann-Whitney U) differed in the group of patients who developed complications. In addition, neither the absolute change in ACE gene transcription following surgery (-0.093 (-0.130-0.094) *vs* -0.001 (-0.198-0.331) IOD, complications *vs* no complications, p=0.223, Mann-Whitney U) nor the percentage change (-26 (-38-45) *vs* 0 (-49-148) %, p=0.368, Mann-Whitney U) was of any significance.

Likewise, pre-operative (206 (167-251) *vs* 178 (127-257) ng/ml, complications *vs* no complications, p=0.220, Mann-Whitney U), post-operative (117 (107-251) *vs* 128 (56-182) ng/ml, p=0.920, Mann-Whitney U) and the change (-60 (-89-0) *vs* -45 (-94-12) ng/ml, p=0.687, Mann-Whitney U) in ACE protein levels were similar in all patients, regardless of whether complications arose.

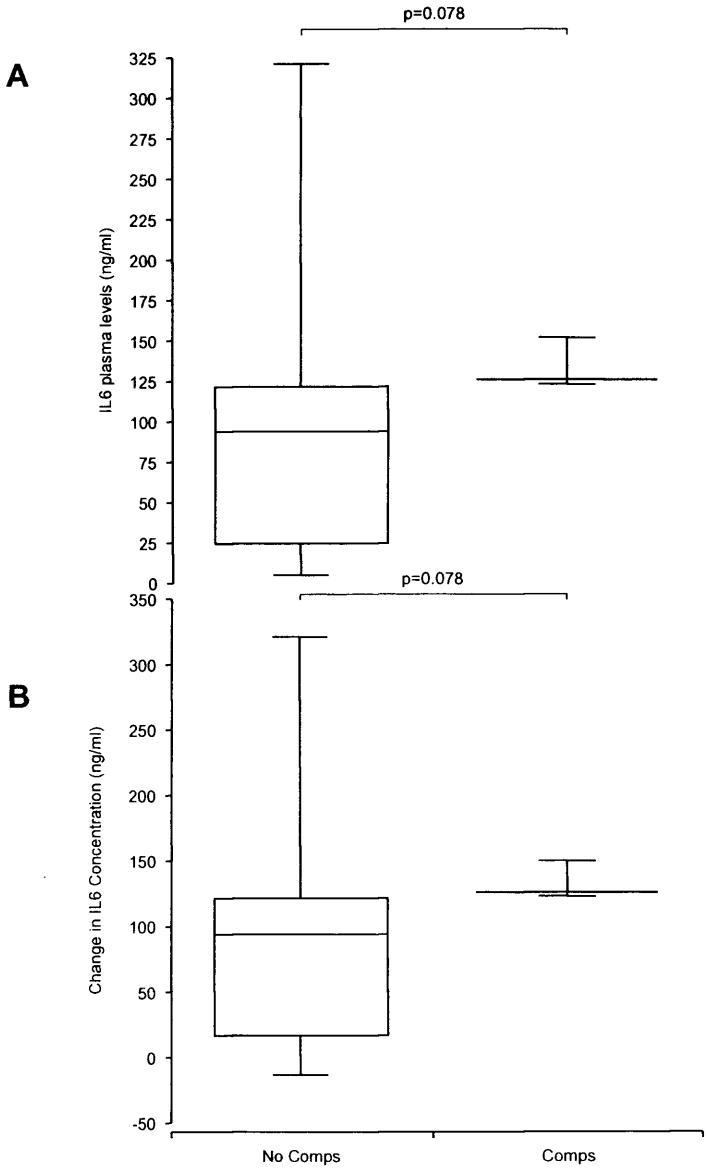


Figure 7.10 Complications by post-operative IL6 protein levels and change in IL6 protein levels

Box and whisker plots representing median (line) values with quartiles (box) and range (error). Comps, complications. There was a tendency for those patients who developed post-operative complications to have **(A)** greater post-operative IL6 plasma protein levels (p=0.078, Mann-Whitney U) and **(B)** to have launched a greater IL6 plasma protein response to surgery (p=0.078, Mann-Whitney U).

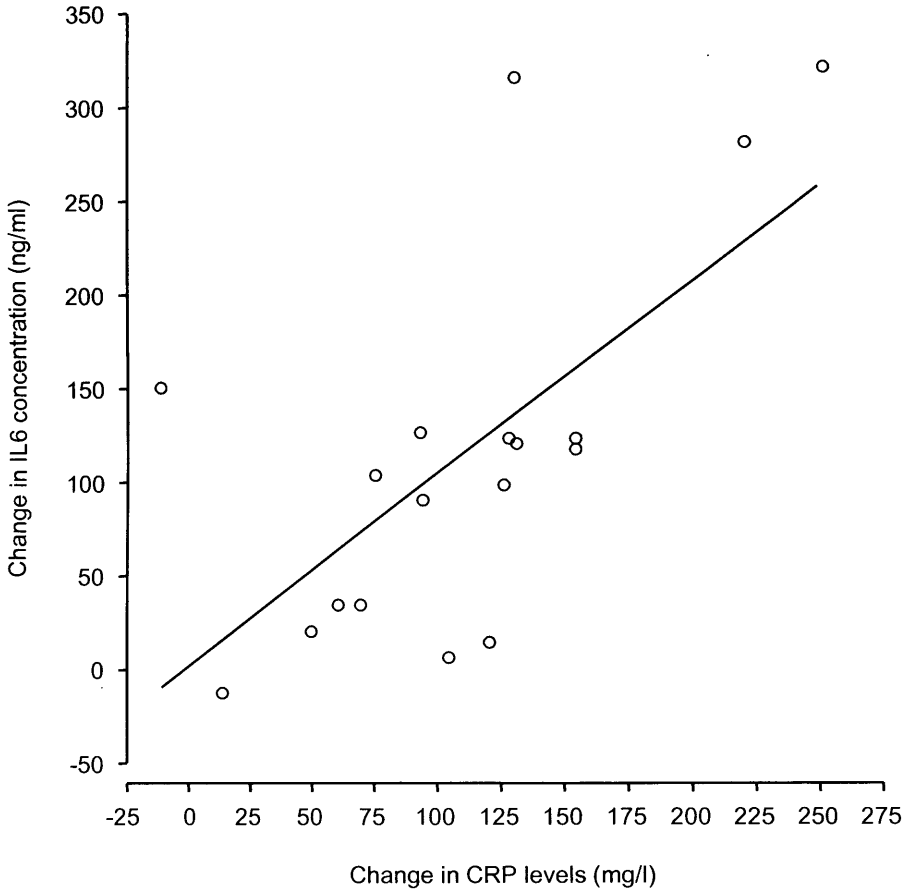


Figure 7.11 Post-operative changes in IL6 protein and plasma CRP levels

Points represent individual patient cases. CRP, C-reactive protein. There was a positive correlation between the post-operative changes in IL6 plasma levels and CRP levels measured at 24 hours following surgery (p=0.017, R=0.533, Spearman's rank correlation).

7.4 DISCUSSION

Considering IL6 first, the possibility that the individual pro-inflammatory response to surgery may have an impact on the post-operative outcome has been much researched, but the underlying role of genetic polymorphisms in determining this reaction has only recently begun to receive attention. The examination of possible genetic predisposition to poorer surgical outcome, based on the -174 G/C genotype has thus far focused on AAA and CABG patients. In both cases, G/G homozygotes have been found to be at greater risk of post-operative complications (Gaudino *et al.,* 2003; Bown *et al.,* 2004). Interestingly, G/G homozygote CABG patients were also found to mount a greater total IL6 protein response to surgery, as measured up to the point of discharge. Moreover, this greater IL6 response also correlated with the development of both renal and pulmonary post-operative complications (Gaudino *et al.,* 2003). On the other hand, despite associating the incidence of organ failure following elective AAA repair with the G-allele, measurement of peri-operative IL6 levels did not provide any such evidence of a functional polymorphic effect. However, in ruptured AAA surgery, elevated post-operative IL6 levels did correlate with the risk of MOF, although in this cohort, no genotypic influence was demonstrated (Bown *et al.,* 2004).

The results presented from this study of colorectal cancer patients undergoing laparotomy were in line with these earlier findings. Thus, as has been found previously, a trend towards IL6 -174 C-allele carriers having a better outcome was observed, and all those patients who experienced problems following surgery were found to be IL6 -174 G/G homozygotes (Figure 7.9). Furthermore, there was again support for the polymorphism being functional; evidence of a positive correlation between pre-operative levels of IL6 gene transcription and LOS was seen (Figure 7.6), and patients

mounting a greater IL6 protein response in the immediate post-operative period were also more likely to develop complications (Figure 7.10).

The significance of these findings is debatable, given the small numbers of patients involved and the limitations of the RT-PCR and ELISA experimental methods employed (see Chapter 6). In fact, such errors may again explain the observed lack of congruity between the influence of the post-operative transcription and protein responses. Alternatively, again this may have been due to the existence of numerous functional IL6 promoter polymorphisms, which act in a co-operative manner (Terry *et al.,* 2000). Therefore, rather than analyse a single polymorphism, such as -174 G/C, in isolation, the assessment of a patient's haplotype clade provide more robust findings. Such an approach has been successfully taken with a study of critically ill adults, which found that haplotype clades rather than individual SNPs associated with increased mortality and organ dysfunction (Sutherland *et al.,* 2005). However, the simultaneous assessment of multiple SNPs was beyond the scope of this study, and would have been difficult to interpret given the small study size. Another possible concern is the fact that the opposite allele-linked effect has also been reported, with C/C homozygotes mounting a bigger IL6 response to CABG surgery. However, this was a comment on the peak post-operative IL6 response, rather than the overall response; any genotypic difference was actually only evident at 6 hours after surgery, and disappeared at later time-points (Brull *et al.,* 2001).

The role played by IL6 as a key co-ordinator, being released in concert with other cytokines such as TNFa and IL1ß in response to a surgical insult, has already been described in Section 1.1. A greater post-operative rise of the pro-inflammatory TNFa

and IL1ß has been related to poorer outcome (Roumen *et al.,* 1993), so it is perhaps unsurprising that an elevated IL6 response had a similar association (Baigrie *et al.,* 1993; Mokart *et al.,* 2002). It is thought that the mechanism underlying this is a combination of an overwhelming pro-inflammatory response, and the ability of IL6 to also stimulate a compensatory anti-inflammatory response (Biffl *et al.,* 1996; and see Section 1.1). Interestingly, the IL6 protein response to surgery positively correlated with the post-operative rise in plasma levels of the well-described and clinically relevant acute phase protein CRP (Figure 7.11). This finding would seem to support the possibility of IL6's pro-inflammatory role underlying the association with poorer post-operative outcome. Moreover, given these results and its prevalence in clinical practice as a non-specific inflammatory marker, the further investigation of CRP as a potential prognostic marker for outcome may be warranted.

Overall, the evidence of association between genotype, IL6 protein response and the development of complications; closer analysis of work that made contrary conclusions (Brull *et al.,* 2001); the existence of several studies reporting poorer outcome associated with a greater IL6 response (Baigrie *et al.,* 1993; Mokart *et al.,* 2002); and the general agreement with the past results from CABG and AAA patients (Gaudino *et al.,* 2003; Bown *et al.,* 2004) still make it likely that the G/C genotype had a functional effect in influencing both IL6 response and subsequent outcome of patients within this study. More specifically, the poorer outcome experienced by G/G homozygotes has been previously reported in both immunologically naïve pre-term neonates (Harding *et al.,* 2003), and adult CABG patients (Gaudino *et al.,* 2003).

However, although a similar association between the G-allele and longer post-operative LOS has been reported in CABG patients (Burzotta *et al.,* 2001), this was not found to be the case here (Figures 7.1 and 7.2). It is unclear why an allelic association with post-operative complications should exist, without also corresponding to LOS. Interestingly, in this study, there was no difference in LOS between those who experienced complications, and those who did not.

The rationale for choosing LOS as an outcome measure, and the role of possible confounding factors in determining it, have been discussed in Chapter 4. Briefly, LOS has been used as an end-point marker of outcome, both medically and financially (Tartter, 1996; Braga *et al.,* 2005), although potential pitfalls such as "social" delay and differences in peri-operative management have been recognised. Some of these medical management decisions, such as drain placement, pace of diet advancement and choice of analgesia may have been responsible for the lack of IL6 genotypic association observed. However, this is unlikely, given that such peri- and post-operative management decisions were unlikely to have varied greatly within the group, due to the relatively homogeneous nature of the patient population, in terms of operation undergone. Moreover, the likelihood of medical decision-making acting as a confounding factor is further lessened by the study setting within the sole colorectal unit of a single institution, with established and consistent staff and management protocols.

Turning to consider ACE, the I/D polymorphism did seem to have influence over surgical outcome, as measured by LOS (Figure 7.3). More specifically, D-allele carriers tended to have a longer post-operative LOS (Figure 7.4). In addition, categorising patients according to whether they had LOS =10 days (short-stayers), revealed a

preponderance of I/I homozygotes compared to the long-stay group (Figure 7.5). Furthermore, despite there being no clear association between ACE genotype and the development of post-operative complications, it was interesting to note that none of these poorer-outcome patients were I/I homozygotes. Although the small patient sample size does make it difficult to make more firm suggestions, again, the relative homogeneity of the population does offset this to some extent.

Nevertheless, this association of the D-allele with poorer surgical outcome has previously been reported in oesophagectomy patients; those with the D/D genotype were roughly three times more likely to develop pulmonary complications following surgery (Lee *et al.,* 2005). Interestingly, despite finding that plasma ACE protein levels increased in a dose-dependent fashion with the presence of the D-allele, no association between elevated pre-operative ACE concentration and an increased risk of pulmonary complications was evident in these oesophagectomy patients (Lee *et al.,* 2005). This measurement of plasma ACE levels was made as an attempt to confirm that the I/D polymorphism was having an actual functional effect on ACE expression, thereby influencing individual predisposition to pulmonary complications. However, restricting such an analysis to the investigation of pre-operative plasma ACE levels seems incomplete. Especially since the cytokine response to surgery, rather than the baseline pre-operative inflammatory state is likely to be the influential factor (Butler *et al.,* 1993).

In this study, despite the smaller number of recruited subjects, a much more complete examination of ACE expression was carried out, seeking a more definitive answer to the question of whether or not any I/D polymorphic influence was mediated by functional

alteration of the ACE gene, or due to linkage disequilibrium with other genes. So, along with evidence pointing towards the D-allele being a marker of poorer surgical outcome, there was also an indication that this was caused by a functional impact on the ACE gene; those who mounted a greater ACE transcriptional response to surgery seemed more likely to have a shorter post-operative LOS (Figure 7.7). Furthermore, the finding that D/D homozygotes launched a greater ACE gene transcription response to surgery (see Chapter 6), provides further evidence for the I/D polymorphic association with outcome being a real rather than spurious result or a marker for other genes. In addition, its multifunctional role as a pro-inflammatory cytokine, endothelial functional mediator, growth factor and fluid homeostasis regulator (see Chapter 1), does makes it possible that the ACE expression response to surgery might influence outcome. Further research is required before the precise nature of this effect becomes clear.

To summarise the main findings from this investigation of the potential influence of the ACE I/D and IL6 -174 G/C genotypes on outcome from colorectal cancer surgery:

- There was no evidence of association between the -174 G/C genotype and LOS, even when patients were divided into "short" and "long-stayers" (Figures 7.1 and 7.2).
- Analysing both the baseline pre-operative state and the response to surgery, there was also no association between IL6 gene transcription, protein expression and LOS.
- However, when assessing outcome in terms of post-operative complications, a trend towards IL6 -174 C-allele carriers having a better outcome was observed (Figure 7.9).

- Similarly, a trend was observed towards patients developing complications having greater post-operative rise in IL6 protein levels (Figure 7.10), although there was no such association with IL6 gene transcription.
- This rise in IL6 protein levels positively correlated with the post-operative change in CRP levels (Figure 7.11).
- Considering the ACE I/D polymorphism, although no obvious association between genotype and LOS was seen (Figure 7.3), there was a trend was towards a longer LOS in patients possessing a D-allele (Figure 7.4), and a preponderance of I/I homozygotes in the "short" stay group (Figure 7.5).
- A negative correlation between the percentage change in ACE gene transcription following surgery and LOS was observed (Figure 7.8), and patients in the "short-stay" group tended to have a greater percentage ACE gene transcription (Figure 7.7). However, no such association between ACE protein expression and LOS was observed.
- There was no association between ACE gene transcription or protein expression and the development of post-operative complications.

CHAPTER 8

Discussion and Summary

CHAPTER 8

The inflammatory response to surgery has been shown to associate with post-operative outcome (Roumen *et al.,* 1993; Table 1.5). Functional genetic polymorphisms affecting inflammatory cytokine genes have been described, and are likely to underlie the basis of individual variation in cytokine response seen in patients following the same inflammatory stimulus (Damas *et al.,* 1997). Furthermore, this variation in response may be a key determinant for differences in surgical outcome. Such a relationship has been reported in CABG, organ transplantation and AAA surgery, however as yet no description has been made for patients undergoing colorectal surgery. Given all of this, the aim of the thesis was to assess the IL6 and ACE response to elective colorectal surgery, in terms of gene activity and levels of protein production, and investigate the influence of the functional I/D ACE and -174 G/C polymorphisms on this reaction and post-operative outcome. The choice of LOS and the incidence of post-operative complications as the outcome measures was in-line with previous reports (Tartter, 1996; Lawrence *et al.,* 1995).

Patient demographics, simple inflammatory markers and outcome

The recruited study population was similar to previously reported series (Fazio *et al.,* 2004), with a median age of 69 years, slight male pre-ponderance, and even distribution of Dukes' stage, allowing the reasonable generalization of any findings. Importantly, all were Caucasian, and underwent elective surgery for colorectal cancer, rather than benign conditions such as ulcerative colitis or diverticulitis, thus limiting the potential for disease-related confounding factors to affect the peri-operative levels of inflammatory cytokines such as IL6, CRP and ACE. In addition, both ACE I/D and IL6

-174 G/C polymorphisms were in Hardy-Weinberg equilibrium (Table 4.6), with no suggestion of a genotypic bias within this colorectal cancer patient cohort.

The need for novel methods of identifying the high-risk surgical patient was highlighted by the lack of association observed between post-operative LOS and factors that are often taken into account, such as age, ASA score, pre-operative Hb, operation length and Dukes' stage (Tables 4.3 and 4.4). So, although the observed rise in established inflammatory markers such as WCC (Figure 4.2) and CRP (Figure 4.3) following surgery was both expected and also previously reported (Sarbinowski *et al.,* 2005; Halevy *et al.,* 1995), the finding of an association between the extent of the WCC rise following surgery and outcome was novel (Figures 4.4 and 4.6).

A greater percentage rise in WCC, as measured 24 hours following surgery, was associated with the development of complications (Figure 4.4), and a prolonged LOS (Figure 4.6). Importantly, none of the patients within this study cohort was taking any anti-inflammatory medication (steroid or non-steroidal). Patients demonstrating an elevated WCC response to surgery may proceed towards a hyper-inflammatory SIRS or paradoxical immunosuppression state (Davies *et al.,* 1997), both of which can increase the risk of post-operative morbidity and ultimately prolong LOS (Novitsky *et al.,* 2004). Despite being a simple barometer of the patient's immune response to the insult of surgery, using the peri-operative change in WCC as a potential prognostic indicator has not previously been reported, and this may form the basis of a larger study into its potential role in post-operative care.

IL6 response to surgery, -174 G/C polymorphism and outcome

As a key pro-inflammatory cytokine, the IL6 response to surgery has been widely investigated, including in colorectal surgery (Miki *et al.,* 2005). So, the observed rise IL6 plasma protein levels at both 4 and 24 hours following surgery (Figure 5.9) was not unexpected. However, this was accompanied by a significant rise in IL6 gene transcription (Figures 5.5 and 5.6), which has not been previously described; previous work has only investigated circulating IL6 protein levels, despite the known short half of this cytokine, and its consequent likely regulation at the level of transcription (Castell *et al.,* 1988). The parallel nature of the post-operative increases in both IL6 gene transcription and protein expression, along with the positive correlation between these two indices at the 24 hour post-operative timepoint (Figure 5.11) would seem to reinforce the conclusion that a real rather than spurious effect was observed. Furthermore, the rise in IL6 plasma protein concentration following surgery correlated positively with the change in plasma levels of the well-established acute phase reactant CRP (Figure 7.11).

The potential significance of this IL6 response to surgery is reflected by the finding that those patients displaying a greater increase in protein expression tended to be more likely to develop post-operative complications (Figure 7.10), although there was no such association with gene transcription. Such an association between poorer outcome and elevated IL6 plasma protein response has previously been reported (Mokart *et al.,* 2002), and may be due to the development of a hyper-inflammatory state and consequent SIRS. The parallel rise in CRP observed in concert with IL6 plasma protein levels partly supports this hypothesis. Moreover, as a test that is in widespread clinical use, CRP may additional provide a surrogate marker for the changes in IL6 plasma

protein levels. In contrast, the baseline pre-operative and post-operative IL6 levels of these indices were not associated with complications following surgery. As for outcome in terms of LOS, this was not associated with IL6 expression at any timepoint.

On the other hand, apart from a trend towards C/C homozygotes having lower levels of IL6 gene transcription post-operatively (Figure 5.4), the -174 G/C polymorphism did not seem to have any association with pre-operative baseline or post-operative or the surgery-induced change in levels of IL6 gene transcription or protein expression. Moreover, there was no association between -174 G/C genotype and outcome, in terms of post-operative LOS (Figures 7.1 and 7.2). There was however a trend observed towards IL6 -174 C-allele carriers being less likely to develop complications (Figure 7.9).

The paucity of significant genotypic association with outcome may have been due to the existence of a number of functional polymorphisms within the IL6 gene, making the haplotype rather than any single polymorphism of importance (Terry *et al.,* 2000). This would be in line with past investigation of critically ill adult patients, where haplotype clades rather than individual SNPs were found to be associated with increased mortality and organ dysfunction (Sutherland *et al.,* 2005). Hence, although this was beyond the scope of this particular study, any future investigation of the genotypic influence over the IL6 response to surgery would have to take this into account.

ACE the I/D polymorphism and the response to surgery, with allelic breakdown

Starting with the pre-operative baseline state, there was initially no significant association between ACE I/D polymorphism, gene transcription (Figure 6.2) and plasma protein levels (Figure 6.14), which was also evident 24 hours following surgery (Figure 6.15). However, considering the gene transcription results from just the ACE 2215 primers, I/I homozygotes had greater levels of transcription pre-operatively (Figure 6.3). The differential allelic contribution to transcription was successfully assessed; in the well controlled situation of investigating I/D heterozygotes, the D-allele was responsible for greater levels of gene transcription (Figures 6.5 and 6.6). This remained a significant finding, even when combining the heterozygote results with those from the rest of the study group - the D-allele was still found to produce a greater degree of transcription (Figure 6.9).

Moving on to the response to colorectal surgery, there was a rise in ACE gene transcription noted 24 hours following surgery (Figure 6.11), which despite the increasingly evident pro-inflammatory role of this molecule, has not previously been reported. When considering just the gene transcription results from the ACE 2215 primers, an association between the scale of this ACE gene transcription response and the I/D polymorphism became evident, with D/D homozygotes producing the greatest response (Figure 6.12). However, no such genotypic relationship with the ACE plasma protein response to surgery was observed. Similarly, investigation of a differential I/D allelic response to surgery did not initially reveal any obvious significant differences. However, the degree to which the D-allele dominated transcription did decrease post-operatively (Figure 6.13), suggesting a greater I-allele response to surgery in I/D heterozygotes, has also not been previously documented.

Conversely, there was a fall in the levels of ACE plasma protein 24 hours following surgery (Figure 6.16), which remained after correction for possible haemodilution effects using the corresponding haematocrit values (Figure 6.18). Again, the degree of this change in ACE expression was unassociated with the I/D polymorphism. Moreover, this post-operative drop in circulating ACE protein concentration has also not previously been reported, although a fall in ACE activity following thoracic surgery has been documented (Karnezis, 1999). The cause of this fall is unclear, and may be due to consumption of the ACE molecule as an inflammatory response is mounted following surgery. This may in turn trigger a negative feedback response, resulting in the compensatory rise in ACE gene transcription that was observed.

Turning to the long term effects of surgery on ACE, both protein expression and gene transcription levels were found to be elevated at 20 weeks following the operation, with the former also being the case at a 10 week interval timepoint (Figures 6.19 and 6.20). This has not previously been reported, although a prolonged activation of the RAS following CABG surgery in D-allele carriers has been demonstrated (Van Geel *et al.*, 2003). The functional significance of this long-term elevation may lie with the potential roles of ACE as a mediator of tumour-related malnutrition and sarcopenia, both leading to weight loss.

ACE, the I/D polymorphism and outcome from surgery

A trend towards ACE D-allele carriers having a longer post-operative LOS was seen in this study cohort (Figure 7.4). In addition, after dividing the study population into "short" and "long-stayers", depending on whether the LOS was =10 days, a preponderance of I/I homozygotes was found in the group with better outcome (Figure

7.5). There was no such allelic or genotypic association with outcome, in terms of the development of complications.

Despite the lack of observed significant association between the ACE I/D genotype and peri-operative gene transcription or protein expression, there was some evidence that the genotypic effect on outcome was a real effect, rather than being spurious. The "short-stay" patients tended to launch a greater ACE gene transcriptional response to surgery (Figure 7.7), and a negative correlation between the magnitude of this response and LOS was observed (Figure 7.8). The mechanism underlying the poorer outcome seen in patients with a greater ACE transcription response remains unclear. Given the pro-inflammatory role of ACE, it may be that the stimulation towards a hyper-inflammatory and SIRS state may be one possibility, but further research is required.

Conclusion

In summary, the aims of the thesis have been systematically addressed in a study population of elective colorectal cancer surgery patients. There were no demographic, pathological or operative factors that seemed to influence outcome following surgery. The only significant finding was that the magnitude of WCC response to surgery was associated with the development of complications and prolonged LOS.

A significant rise in both IL6 gene transcription and protein levels following surgery was observed, and there was some evidence that the -174 G/C polymorphism was associated with the gene response and post-operative outcome.

Similarly a post-operative elevation in ACE gene transcription was noted, with some evidence that the I-allele was responsible for a greater proportion of this rise; moreover, a greater rise was associated with poorer outcome. In contrast, a fall in ACE protein levels was noted following surgery. Finally, although the ACE I/D polymorphism was not significantly associated with peri-operative gene transcription or plasma protein levels, it nonetheless did seem to affect outcome.

This work further strengthens the previous observations that an assessment of patient genotype may be of importance during the pre-operative work-up, enabling the improved identification of the high-risk surgical patient.

LIST OF PUBLICATIONS

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Lee JT, Chaloner EJ, Hollingsworth SJ. The role of cardiopulmonary fitness and its genetic influences on surgical outcomes. *Br J Surg* 2006; **93**: 147-57

LIST OF PRESENTATIONS

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International

1. Plasma angiotensin-1-converting enzyme (ACE) and its role in colorectal cancer. (Oral)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Royal Society of Medicine (Section of Coloproctology) Meeting (Prague, Czech Republic, June 2006)

National

1. Outcome following colorectal cancer surgery may be influenced by surgery-induced long-term elevation of plasma angiotensin-1-converting enzyme. (Poster)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Association of Surgeons of Great Britain and Ireland Annual Scientific Meeting (Edinburgh, May 2006)

2. The deletion (D) allele of the angiotensin-1-converting enzyme (ACE) polymorphism confers greater ACE expression than the insertion (I) allele, but is not preferentially activated by surgery for colorectal cancer. (Poster)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Association of Surgeons of Great Britain and Ireland Annual Scientific Meeting (Edinburgh, May 2006)

3. The D-allele of the angiotensin-1-converting enzyme (ACE) gene and enhanced ACE gene transcription correlate with increased post-operative stay after surgery for colorectal cancer. (Poster)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Association of Coloproctology of Great Britain and Ireland Annual Scientific Meeting (Gateshead, July 2006)

4. Peri-operative Interleukin-6 (IL6) in patients having surgery for colorectal cancer; is the -174 G/C genotype important? (Poster)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Association of Coloproctology of Great Britain and Ireland Annual Scientific Meeting (Gateshead, July 2006)

5. The deletion (D) allele of the angiotensin-1-converting enzyme (ACE) polymorphism confers greater ACE expression than the insertion (I) allele, but is not preferentially activated by surgery for colorectal cancer. (Poster)

   JT Lee, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth

   Association of Coloproctology of Great Britain and Ireland Annual Scientific Meeting (Gateshead, July 2006)

LIST OF ABSTRACTS

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1. **JT Lee**, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth (2006). The D-allele of the angiotensin-1-converting enzyme (ACE) gene and enhanced ACE gene transcription correlate with increased post-operative stay after surgery for colorectal cancer. *Colorectal Dis*, **8** (Suppl. 2): 34-98

2. **JT Lee**, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth (2006). Peri-operative Interleukin-6 (IL6) in patients having surgery for colorectal cancer; is the -174 G/C genotype important? *Colorectal Dis,* **8** (Suppl. 2): 34-98

3. **JT Lee**, P Ng, EJ Chaloner, PB Boulos, SJ Hollingsworth (2006). The deletion (D) allele of the angiotensin-1-converting enzyme (ACE) polymorphism confers greater ACE expression than the insertion (I) allele, but is not preferentially activated by surgery for colorectal cancer. *Colorectal Disease Dis,* **8** (Suppl. 2): 34-98

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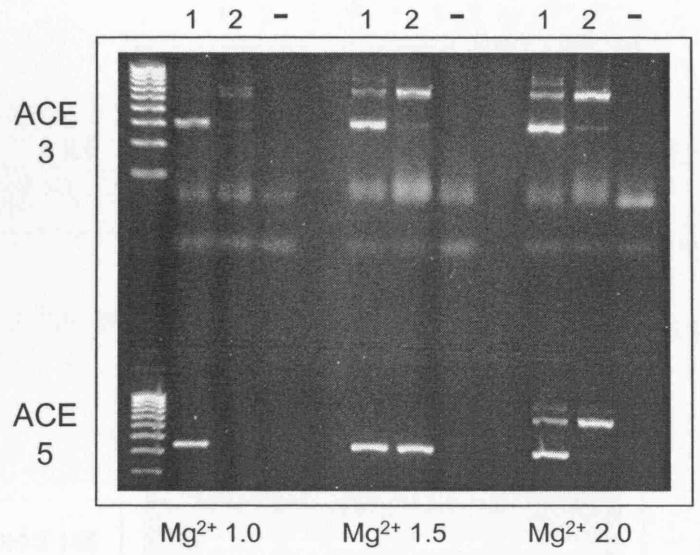
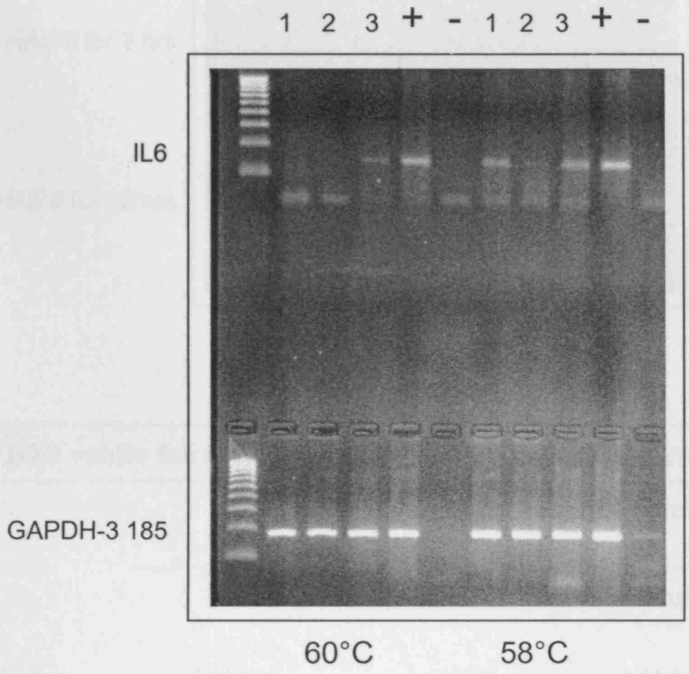
Appendix I

Optimisation of experimental protocols



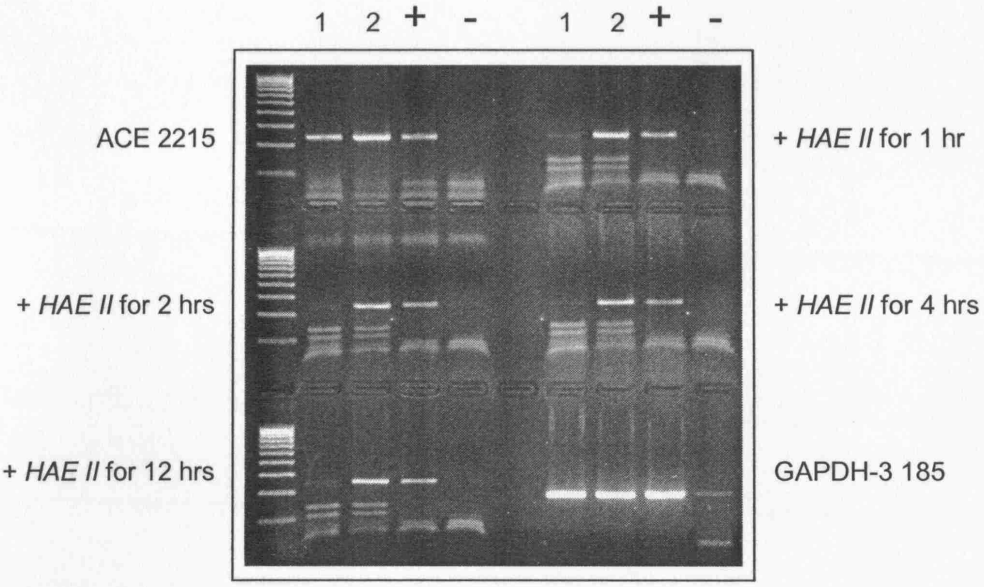
Results for ACE genotype PCR protocol optimisation - Mg^{2+} concentration titration

Polaroid™ photograph following agarose gel electrophoresis separation of PCR products with both H ACE 3 and H ACE 5 primers at different Mg^{2+} reaction concentrations. 1,2, Patient samples. -, Negative control. At Mg^{2+} 1.0mM, sample 2 had no product with H ACE 5 primers – the reaction was too stringent. At Mg^{2+} 2.0mM, there were extra PCR product bands from non-specific amplification – the reaction not stringent enough. At Mg^{2+} 1.5mM optimal stringency was observed – Patient 1 was an I/D heterozygote and Patient 2 a I/I homozygote. Similar processes of Mg^{2+} concentration titration were carried out for all the PCR and RT-PCR protocols.



Results for IL6 RT-PCR protocol optimisation - annealing temperature titration

Polaroid™ photograph following agarose gel electrophoresis separation of RT-PCR products with IL6 primers at different annealing temperatures. 1,2,3, Patient samples. +, Positive control -, Negative control. At an annealing temperature of 60°C, there were no product bands for patients 1 & 2 - the reaction was too stringent. However at an annealing temperature of 58°C, product bands of the expected size were evident for all patients – the reaction stringency was improved. Similar processes of annealing temperature titration were carried out for all the PCR and RT-PCR protocols.



IOD values for *HAE II* restriction reaction of different durations

	Restriction reaction duration			
	1 hr	2 hrs	6 hrs	12 hrs
Pre-restriction	141000	141000	141000	141000
Post-restriction	133268	110144	105708	88186
Ratio	0.945	0.781	0.750	0.625

Results for *HAE II* restriction reaction optimisation

Polaroid™ photograph following agarose gel electrophoresis separation of RT-PCR and *HAE II* restriction products. 1,2, Patient samples. +, Positive control. -, Negative control. **IOD** – Integrated optical density results; **Pre-restriction** – IOD results before the restriction reaction; **Post-restriction** – IOD results following the restriction enzyme; **Ratio** – ratio of the pre- *vs* post-restriction IOD results. There was no difference between 2 hours and 12 hours of restriction enzyme efficacy. A similar process was carried out for the *NLA III* restriction enzyme reaction within the IL6 genotyping protocol.

Appendix II

Patient demographics, genotype, operation and outcome data

Patient Demographic and Pathology Data

Sample ID	Age	Sex	ASA Score	Dukes' Stage	Sample ID	Age	Sex	ASA Score	Dukes' Stage
Lap 001	65	F	2	B	**Lap 020**	78	M	2	B
Lap 004	81	F	2	C	**Lap 021**	72	M	2	C
Lap 005	77	F	2	C	**Lap 022**	76	M	2	A
Lap 006	80	M	3	C	**Lap 023**	61	M	1	A
Lap 007	67	M	3	B	**Lap 024**	47	M	1	C
Lap 008	79	M	3	A	**Lap 025**	69	F	2	C
Lap 009	87	M	2	B	**Lap 026**	58	F	2	C
Lap 010	68	M	2	C	**Lap 027**	67	F	2	A
Lap 011	55	F	2	B	**Lap 028**	69	F	2	B
Lap 012	73	M	2	C	**Lap 031**	67	M	2	A
Lap 014	81	M	2	B	**Lap 032**	68	M	2	B
Lap 015	43	M	2	C	**Lap 033**	81	M	2	C
Lap 017	69	F	2	A	**Lap 034**	77	F	3	A
Lap 018	73	M	2	B	**Lap 035**	60	F	2	B
Lap 019	76	F	2	B					

Demographic details for study patients. **Sample ID** – sample identification number; **Age**– age of patient in years; **Sex** – male (M) or female (F); **ASA** – American Society of Anesthesiology score (1-4)

Patient Genotype and Operative Details

Sample ID	Genotype		Operation		Sample ID	Genotype		Operation	
	ACE	IL6	Procedure	Time		ACE	IL6	Procedure	Time
Lap 001	I/I	G/C	Lt Hemi	189	**Lap 020**	I/D	G/G	Rt Hemi	142
Lap 004	D/D	G/G	Ant Resection	175	**Lap 021**	I/D	G/C	Sigmoid	101
Lap 005	I/D	C/C	Rt Hemi	247	**Lap 022**	I/I	G/G	Rt Hemi	126
Lap 006	I/I	C/C	Ant Resection	227	**Lap 023**	I/D	G/C	Ant Resection	313
Lap 007	I/D	G/G	Subtotal	205	**Lap 024**	I/D	G/G	A-P Resection	471
Lap 008	I/D	G/C	Ant Resection	207	**Lap 025**	I/I	G/C	Rt Hemi	176
Lap 009	I/D	G/G	Rt Hemi	193	**Lap 026**	I/D	G/C	Rt Hemi	252
Lap 010	I/D	G/G	Ant Resection	235	**Lap 027**	I/I	G/G	Subtotal	270
Lap 011	D/D	G/G	Lt Hemi	316	**Lap 028**	I/I	G/C	Ant Resection	297
Lap 012	D/D	C/C	Lt Hemi	241	**Lap 031**	D/D	G/C	Ant Resection	210
Lap 014	I/D	C/C	Lt Hemi	349	**Lap 032**	I/D	G/C	Ant Resection	215
Lap 015	I/D	G/C	A-P Resection	339	**Lap 033**	D/D	G/G	Ant Resection	177
Lap 017	I/D	G/G	Lt Hemi	181	**Lap 034**	D/D	G/G	Sigmoid	235
Lap 018	I/I	G/G	Rt Hemi	203	**Lap 035**	I/D	G/G	Ant resection	255
Lap 019	D/D	G/G	Lt Hemi	271					

Patient genotype and operative details. **Sample ID** – sample identification number; **Operation Procedure** – type of operation undergone; **Operation time** – length of operation in minutes (including anaesthetic time)

Patient Outcome Data					
Sample ID	**LOS (days)**	**Complications**	**Sample ID**	**LOS**	**Complications**
Lap 001	10	-	**Lap 020**	19	-
Lap 004	25	-	**Lap 021**	15	-
Lap 005	9	-	**Lap 022**	9	-
Lap 006	15	-	**Lap 023**	10	-
Lap 007	31	-	**Lap 024**	15	Wound infection
Lap 008	11	-	**Lap 025**	8	-
Lap 009	11	Wound infection	**Lap 026**	14	-
Lap 010	19	-	**Lap 027**	17	-
Lap 011	8	-	**Lap 028**	11	-
Lap 012	11	-	**Lap 031**	13	-
Lap 014	14	-	**Lap 032**	11	-
Lap 015	25	-	**Lap 033**	9	-
Lap 017	11	-	**Lap 034**	21	Cardiac failure
Lap 018	8	-	**Lap 035**	10	-
Lap 019	17	-			

Patient outcome data. **Sample ID** – sample identification number; **LOS** – Length of stay post-operation (days); **Complications** – Presence or absence of post-operative complications

Appendix III

Peri-operative blood results

Peri-operative Blood Tests								
Sample ID	Pre-operative				Post-operative			
	WCC ($x10^9$)	Hb (g/dl)	Hct	CRP (mg/l)	WCC ($x10^9$)	Hb (g/dl)	Hct	CRP (mg/l)
Lap 001	6.9	10.7	0.324	2.5	9.8	8.4	0.254	-
Lap 004	7.0	12.9	0.391	1.5	13.5	11.3	0.343	-
Lap 005	6.4	9.8	0.313	26.9	8.1	10.6	0.331	-
Lap 006	7.7	13.2	0.399	12.0	9.3	9.2	0.279	-
Lap 007	11.5	8.9	0.291	271.3	12.4	9.8	0.302	-
Lap 008	5.0	12.8	0.410	0.9	9.7	10.6	0.331	71.1
Lap 009	7.8	10.0	0.327	27.1	12.9	8.3	0.269	16.4
Lap 010	7.8	11.3	0.358	18.5	13.0	11.9	0.363	139.4
Lap 011	7.6	13.1	0.392	1.4	5.8	8.2	0.244	106.4
Lap 012	4.7	13.5	0.407	1.1	6.4	10.1	0.311	92.2
Lap 014	8.9	13.8	0.407	2.1	12.4	11.0	0.327	97.1
Lap 015	5.1	11.6	0.349	4.5	5.9	7.1	0.216	
Lap 017	4.8	13.6	0.422	0.4	13.0	10.9	0.339	
Lap 018	8.9	12.2	0.390	1.3	9.4	10.1	0.333	156.0
Lap 019	8.7	11.9	0.371	-	10.6	9.8	0.304	
Lap 020	11.9	10.2	0.332	114.5	13.8	10.4	0.329	128.1
Lap 021	7.1	11.7	0.378	1.4	9.6	10.8	0.362	128.3
Lap 022	9.3	8.1	0.266	228.8	12.6	9.7	0.324	279.2
Lap 023	8.2	13.3	0.409	1.7	12.4	10.9	0.335	133.4
Lap 024	2.9	13.0	0.387	1.1	4.7	9.1	0.271	94.6
Lap 025	9.1	10.6	0.360	95.8	12.4	8.4	0.276	171.4
Lap 026	9.5	11.8	0.378	8.1	16.3	10.1	0.323	138.9
Lap 027	7.1	14.3	0.430	3.3	7.4	11.0	0.343	224.6
Lap 028	8.8	14.2	0.437	1.4	10.1	11.4	0.358	156.4
Lap 031	12.0	14.1	0.407	17.2	8.1	9.0	0.264	269.1
Lap 032	6.4	14.0	0.426	1.0	11.5	11.7	0.367	
Lap 033	10.0	13.6	0.415	1.4	10.4	8.2	0.248	
Lap 034	7.8	10.4	0.347	1.0	12.7	7.3	0.250	129.7
Lap 035	5.6	11.7	0.361	-	6.4	9.3	0.292	144.2

Peri-operative blood test results – pre-operation and 24 hours post-operation. **Sample ID** – sample identification number; **WCC** – White Cell Count ($x10^9$); **Hb** – Haemaglobin level (g/dl); **Hct** – Haematocrit level; **CRP** – C-Reactive Protein level (g/dl)

Percentage/Absolute Changes Following Surgery									
Sample ID	**WCC (%)**	**Hb (%)**	**Hct (%)**	**CRP (mg/l)**	**Sample ID**	**WCC (%)**	**Hb (%)**	**Hct (%)**	**CRP (mg/l)**
Lap 001	41.5	21.5	-21.6	-	**Lap 020**	15.9	-2.0	-0.9	13.6
Lap 004	93	12.4	-12.3	-	**Lap 021**	34.5	7.7	-4.2	126.9
Lap 005	25.7	-8.2	5.8	-	**Lap 022**	35.9	-19.8	21.8	50.4
Lap 006	21.7	30.3	-30.1	-	**Lap 023**	51.4	18.0	-18.1	131.7
Lap 007	7.8	-10.1	3.8	-	**Lap 024**	62.6	30.0	-30	93.5
Lap 008	96.6	17.2	-19.3	70.0	**Lap 025**	36.2	20.8	-23.3	75.6
Lap 009	65.8	17.0	-17.7	-11.0	**Lap 026**	71	14.4	-14.6	130.8
Lap 010	67.2	-5.3	1.4	121.1	**Lap 027**	4.7	23.1	-20.2	221.3
Lap 011	-23.7	37.4	-37.8	105.0	**Lap 028**	15.2	19.7	-18.1	155.0
Lap 012	34.2	25.2	-23.6	61.1	**Lap 031**	-32.5	36.2	-35.1	251.9
Lap 014	39.8	20.3	-19.7	95.0	**Lap 032**	79.2	16.4	-13.8	-
Lap 015	15.1	38.8	-38.1	-	**Lap 033**	3.7	39.7	-40.2	-
Lap 017	174.5	19.9	-19.7	-	**Lap 034**	62.5	29.8	-28	128.7
Lap 018	6.3	17.2	-14.6	154.7	**Lap 035**	13.5	20.5	-19.1	-
Lap 019	22	17.6	-18.1	-					

Percentage or absolute changes in blood test results 24 hours post-operation. **Sample ID** – sample identification number; **WCC** – White Cell Count; **Hb** – Haemaglobin; **Hct** – Haematocrit; **CRP** – C-Reactive Protein level

Appendix IV

Gene transcription

Pre-operative ACE 216 Transcription					
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**IOD (4)**	**Mean IOD**
Lap 001	0.379	0.275	-	-	0.327
Lap 004	0.178	0.071	-	-	0.124
Lap 005	0.090	0.000	-	-	0.045
Lap 006	0.029	0.123	-	-	0.076
Lap 007	0.046	0.061	-	-	0.054
Lap 008	0.118	0.036	-	-	0.077
Lap 009	0.418	0.276	-	-	0.347
Lap 010	0.236	0.095	-	-	0.166
Lap 011	0.282	0.052	-	-	0.167
Lap 012	-	-	-	-	-
Lap 014	0.010	0.111	-	-	0.061
Lap 015	0.019	0.091	-	-	0.055
Lap 017	0.012	0.038	-	-	0.025
Lap 018	0.068	0.019	-	-	0.044
Lap 019	0.083	0.538	0.172		0.264
Lap 020	0.183	0.296	-	-	0.240
Lap 021	0.339	0.082	-	-	0.211
Lap 022	0.093	0.112	-	-	0.103
Lap 023	0.143	0.063	0.286	0.086	0.144
Lap 024	0.165	0.098	0.094	0.137	0.124
Lap 025	0.063	0.100	0.053	0.109	0.081
Lap 026	0.199	0.147	-	-	0.173
Lap 027	0.087	0.878	-	-	0.482
Lap 028	0.187	0.210	-	-	0.198
Lap 031	0.476	0.383	-	-	0.429
Lap 032	0.183	0.114	-	-	0.149
Lap 033	0.045	0.026	-	-	0.035
Lap 034	0.067	0.577	-	-	0.322
Lap 035	-	-	-	-	-

Pre-operative ACE transcription using ACE 216 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-4)** – Integrated optical density, with experimental results from runs 1-4; **Mean IOD** – Mean integrated optical density from all runs

Pre-operative ACE 2215 Transcription									
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**Mean IOD**	**Sample ID**	**IOD (1)**	**IOD (2)**	**IOD (3)**	**Mean IOD**
Lap 001	0.692	0.283	-	0.488	**Lap 020**	0.672	0.376	0.330	0.459
Lap 004	0.380	0.027	-	0.203	**Lap 021**	0.652	0.223	0.350	0.408
Lap 005	0.104	-	-	0.104	**Lap 022**	0.380	0.627	0.707	0.571
Lap 006	0.097	0.326	-	0.212	**Lap 023**	0.431	0.587	-	0.509
Lap 007	0.072	0.075	-	0.071	**Lap 024**	0.154	0.441	-	0.298
Lap 008	0.260	0.174	-	0.217	**Lap 025**	0.157	0.423	-	0.290
Lap 009	0.541	0.204	-	0.373	**Lap 026**	0.969	0.564	0.371	0.635
Lap 010	0.804	0.309	-	0.557	**Lap 027**	0.614	0.411	-	0.512
Lap 011	0.124	0.183	-	0.153	**Lap 028**	0.461	0.494	-	0.477
Lap 012	-	-	-	-	**Lap 031**	0.064	0.129	0.036	0.076
Lap 014	0.240	0.995	0.678	0.637	**Lap 032**	0.032	0.183	-	0.108
Lap 015	0.246	0.799	-	0.522	**Lap 033**	0.049	-	-	0.049
Lap 017	0.424	0.415	-	0.420	**Lap 034**	0.644	0.083	-	0.363
Lap 018	0.719	0.090	-	0.405	**Lap 035**				
Lap 019	0.174	0.091	0.305	0.190					

Pre-operative ACE transcription using ACE 2215 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs

Pre-operative I/D Transcription Breakdown									
Sample ID	D-allele			I allele			D:I allele ratio		
	IOD (1)	IOD (2)	IOD (3)	IOD (1)	IOD (2)	IOD (3)	(1)	(2)	(3)
Lap 008	0.170	0.279	-	0.045	0.106	-	3.773	2.636	-
Lap 009	0.222	0.082	-	0.050	0.049	-	4.447	1.675	-
Lap 010	0.187	0.080	-	0.042	0.042	-	4.424	1.894	-
Lap 013	0.145	0.686	-	0.042	0.265	-	3.442	2.588	-
Lap 014	0.042	0.301	0.677	0.073	0.062	0.350	0.572	4.835	1.938
Lap 015	0.112	0.657	-	0.056	0.085	-	2.024	7.766	-
Lap 017	0.426	0.489	-	0.086	0.236	-	4.976	2.069	-
Lap 020	0.697	0.367	0.394	0.228	0.259	0.146	9.998	1.418	2.910
Lap 021	0.495	0.234	0.370	0.115	0.059	0.187	4.310	3.959	1.984
Lap 023	0.522	0.459	-	0.127	0.215	-	4.108	2.133	-
Lap 024	0.243	0.449	-	0.218	0.417	-	1.114	1.078	-
Lap 026	1.053	0.286	0.477	0.405	0.104	0.118	6.558	2.766	4.878
Lap 032	0.021	0.043	-	0.014	0.026	-	1.533	1.660	-

Pre-operative I/D ACE transcription breakdown – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **D-allele IOD (1-3)** – Integrated optical density from the D-allele, with experimental results from runs 1-3; **I-allele IOD (1-3)** – Integrated optical density from the I-allele, with experimental results from runs 1-3; **D:I allele ratio (1-3)** – ratio of IOD results from both alleles, with experimental results from runs 1-3

Pre-operative I/D Transcription Breakdown

Sample ID	D-allele			I allele			D:I allele ratio		
	IOD (1)	IOD (2)	IOD (3)	IOD (1)	IOD (2)	IOD (3)	(1)	(2)	(3)
Lap 008	13757	31773	-	3646	12052	-	3.773	2.636	-
Lap 009	19756	9542	-	4443	5696	-	4.447	1.675	-
Lap 010	27746	9470	-	6272	4999	-	4.424	1.894	-
Lap 013	17817	82992	-	5176	32073	-	3.442	2.588	-
Lap 014	4999	25885	81949	8732	5354	42294	0.572	4.835	1.938
Lap 015	13711	59763	-	6774	7695	-	2.024	7.766	-
Lap 017	38572	60172	-	7751	29084	-	4.976	2.069	-
Lap 020	56700	45092	15676	5671	31801	5387	9.998	1.418	2.910
Lap 021	42373	27335	44059	9831	6904	22206	4.310	3.959	1.984
Lap 023	61598	49090	-	14993	23018	-	4.108	2.133	-
Lap 024	29624	35887	-	26594	33305	-	1.114	1.078	-
Lap 026	38421	22698	14589	5859	8206	2991	6.558	2.766	4.878
Lap 032	2267	4402	-	1479	2652	-	1.533	1.660	-

Pre-operative I/D ACE transcription breakdown. **Sample ID** – sample identification number; **D-allele IOD (1-3)** – Integrated optical density from the D-allele, not normalised, with experimental results from runs 1-3; **I-allele IOD (1-3)** – Integrated optical density from the I-allele, not normalised, with experimental results from runs 1-3; **D:I allele ratio (1-3)** – ratio of IOD results from both alleles, with experimental results from runs 1-3

Pre-operative I/D Transcription Breakdown Summary			
Sample ID	**D-allele Mean IOD**	**I-allele Mean IOD**	**Mean D:I allele ratio**
Lap 008	0.224	0.075	3.205
Lap 009	0.152	0.050	3.061
Lap 010	0.134	0.042	3.159
Lap 013	0.415	0.154	3.015
Lap 014	0.340	0.162	2.448
Lap 015	0.385	0.070	4.895
Lap 017	0.458	0.161	3.523
Lap 020	0.486	0.211	4.775
Lap 021	0.366	0.120	3.418
Lap 023	0.490	0.171	3.121
Lap 024	0.346	0.317	1.096
Lap 026	0.606	0.209	4.734
Lap 032	0.032	0.020	1.596

Pre-operative I/D ACE transcription breakdown summary – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **D-allele Mean IOD** – Mean integrated optical density from the D-allele, from experimental runs 1-3; **I-allele Mean IOD** – Mean integrated optical density from the I-allele, from experimental runs 1-3; **D:I allele ratio** – Mean ratio of IOD results from both alleles, from experimental runs 1-3

Pre-operative IL6 Transcription

Sample ID	IOD (1)	IOD (2)	IOD (3)	Mean IOD	Sample ID	IOD (1)	IOD (2)	IOD (3)	Mean IOD
Lap 001	0.145	0.069	-	0.107	**Lap 020**	0.128	0.136	-	0.132
Lap 004	0.298	0.124	-	0.211	**Lap 021**	0.210	0.040	-	0.125
Lap 005	0.170	0.102	-	0.136	**Lap 022**	0.155	0.307	-	0.231
Lap 006	0.131	0.163	-	0.147	**Lap 023**	0.263	0.037	0.073	0.124
Lap 007	0.280	0.276	-	0.278	**Lap 024**	0.147	0.041	0.062	0.083
Lap 008	0.070	0.061	-	0.066	**Lap 025**	0.498	0.033	0.188	0.240
Lap 009	0.185	-	-	0.185	**Lap 026**	0.077	0.429	-	0.253
Lap 010	0.035	0.080	-	0.057	**Lap 027**	0.269	0.312	-	0.291
Lap 011	0.122	0.083	-	0.103	**Lap 028**	0.055	0.092	-	0.073
Lap 012	-	-	-	-	**Lap 031**	0.092	0.111	0.155	0.119
Lap 014	0.119	0.046	-	0.082	**Lap 032**	0.086	0.126	-	0.106
Lap 015	0.169	0.064	-	0.116	**Lap 033**	0.033	-	-	0.033
Lap 017	0.055	0.083	-	0.069	**Lap 034**	0.336	0.428	0.308	0.357
Lap 018	0.062	0.124	-	0.093	**Lap 035**	-	-	-	-
Lap 019	0.216	0.101	-	0.159					

Pre-operative IL6 transcription – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs

Transcription 4 hours Post-operation				
ACE 216				
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**Mean IOD**
Lap 011	0.048	0.016	-	0.032
Lap 012	0.036	0.031	-	0.034
Lap 014	0.049	0.059	-	0.054
Lap 020	0.424	-	-	0.424
ACE 2215				
Lap 008	0.237	-	-	0.237
Lap 010	0.578	0.262	-	0.420
Lap 011	0.565	0.769	0.572	0.635
Lap 012	0.457	0.058	0.071	0.195
Lap 014	0.124	0.029	0.381	0.178
Lap 020	0.068	0.257	-	0.163
IL6				
Lap 008	0.164	-	-	0.164
Lap 010	0.097	0.037	-	0.067
Lap 011	0.101	0.051	-	0.076
Lap 012	0.051	0.090	-	0.070
Lap 014	0.049	0.577	-	0.313
Lap 020	0.367	1.319	-	0.843

Transcription 4 hours post-operation for ACE 216, ACE 2215 and IL6 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs

ACE 216 Transcription 24 hours Post-operation					
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**IOD (4)**	**Mean IOD**
Lap 008	0.071	-	-	-	0.071
Lap 009	0.267	0.420	-	-	0.343
Lap 010	0.025	0.079	-	-	0.052
Lap 011	0.041	0.089	-	-	0.065
Lap 012	0.158	0.319	-	-	0.238
Lap 014	0.096	0.074	-	-	0.085
Lap 017	0.150	0.650	-	-	0.400
Lap 018	0.081	0.396	-	-	0.239
Lap 019	0.088	0.032	-	-	0.060
Lap 020	0.287	0.185	-	-	0.236
Lap 021	0.268	0.539	0.119	-	0.309
Lap 022	0.301	0.211	-	-	0.256
Lap 023	0.066	0.114	0.040	-	0.073
Lap 024	0.131	0.025	-	-	0.078
Lap 025	0.097	0.203	0.144	0.176	0.155
Lap 026	0.101	0.044	-	-	0.072
Lap 027	0.341	0.307	-	-	0.324
Lap 028	0.113	0.258	-	-	0.185
Lap 031	0.305	0.365	0.143	0.118	0.233
Lap 032	0.066	0.070	0.081	-	0.072
Lap 034	0.200	0.438	-	-	0.319

ACE transcription 24 hours post-operation using ACE 216 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-4)** – Integrated optical density, with experimental results from runs 1-4; **Mean IOD** – Mean integrated optical density from all runs

ACE 2215 Transcription 24 hours Post-operation				
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**Mean IOD**
Lap 008	0.237	0.514	-	0.375
Lap 009	0.172	0.208	-	0.190
Lap 010	1.173	0.789	-	0.981
Lap 011	0.661	0.462	0.658	0.594
Lap 012	1.166	0.050	0.915	0.710
Lap 014	0.304	0.468	0.861	0.544
Lap 017	1.074	0.231	0.644	0.650
Lap 018	1.563	0.414	0.638	0.872
Lap 019	0.050	0.266	0.225	0.180
Lap 020	0.207	0.662	0.286	0.385
Lap 021	0.658	1.071	0.573	0.767
Lap 022	0.320	0.489	0.439	0.416
Lap 023	0.230	0.750	-	0.490
Lap 024	0.529	0.531	-	0.530
Lap 025	0.408	0.829	-	0.619
Lap 026	0.396	0.332	0.290	0.339
Lap 027	0.483	0.517	-	0.500
Lap 028	0.419	0.313	-	0.366
Lap 031	0.147	0.977	0.057	0.394
Lap 032	0.057	0.096	-	0.076
Lap 034	0.158	0.053	-	0.106

ACE transcription 24 hours post-operation using ACE 2215 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs

IL6 Transcription 24 hours Post-operation				
Sample ID	**IOD (1)**	**IOD (2)**	**IOD (3)**	**Mean IOD**
Lap 008	0.074	-	-	0.074
Lap 009	0.699	-	-	0.699
Lap 010	0.147	-	-	0.147
Lap 011	0.030	0.094	-	0.062
Lap 012	0.081	0.025	-	0.053
Lap 014	0.079	0.116	-	0.098
Lap 017	0.079	0.129	0.103	0.103
Lap 018	0.088	0.819	-	0.454
Lap 019	0.085	0.097	-	0.091
Lap 020	0.313	0.927	-	0.620
Lap 021	0.104	0.276	-	0.190
Lap 022	0.242	0.312	-	0.277
Lap 023	0.436	0.114	-	0.275
Lap 024	0.394	0.069	-	0.231
Lap 025	0.394	0.133	-	0.263
Lap 026	0.151	0.545	-	0.348
Lap 027	0.146	0.250	-	0.198
Lap 028	0.067	0.334	-	0.200
Lap 031	0.058	0.335	0.045	0.146
Lap 032	0.027	0.490	-	0.258
Lap 034	0.243	0.071	-	0.157

ACE transcription 24 hours post-operation using ACE 2215 primers – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs

4 hour Post-operative I/D Transcription Breakdown Summary			
Sample ID	**D-allele Mean IOD**	**I-allele Mean IOD**	**Mean D:I allele ratio**
Lap 008	0.335	0.170	1.969
Lap 010	0.322	0.068	5.476
Lap 014	0.073	0.078	0.875
Lap 020	0.212	0.114	2.236
24 hour Post-operative I/D Transcription Breakdown Summary			
Lap 008	0.221	0.117	1.723
Lap 009	0.087	0.043	2.155
Lap 010	0.963	0.270	3.736
Lap 014	0.192	0.125	1.909
Lap 017	0.380	0.263	1.941
Lap 020	0.401	0.210	2.664
Lap 021	0.587	0.194	3.887
Lap 023	0.284	0.212	1.375
Lap 024	0.446	0.273	1.630
Lap 026	0.338	0.139	3.623
Lap 032	0.046	0.031	1.502

Post-operative I/D ACE transcription breakdown summary at 4 hours and 24 hours post-operation – all results normalised to GAPDH-3. **Sample ID** – sample identification number; **D-allele Mean IOD** – Mean integrated optical density from the D-allele, from experimental runs 1-3; **I-allele Mean IOD** – Mean integrated optical density from the I-allele, from experimental runs 1-3; **D:I allele ratio** – Mean ratio of IOD results from both alleles, from experimental runs 1-3

Overall Mean ACE Transcription (IOD)							
Sample ID	**Pre-op**	**4 hours**	**24 hours**	**Sample ID**	**Pre-op**	**4 hours**	**24 hours**
Lap 001	0.407	-	-	**Lap 020**	0.350	0.293	0.310
Lap 004	0.164	-	-	**Lap 021**	0.309	-	0.538
Lap 005	0.075	-	-	**Lap 022**	0.337	-	0.336
Lap 006	0.144	-	-	**Lap 023**	0.327	-	0.281
Lap 007	0.063	-	-	**Lap 024**	0.211	-	0.304
Lap 008	0.147	0.237	0.223	**Lap 025**	0.186	-	0.387
Lap 009	0.360	-	0.267	**Lap 026**	0.404	-	0.206
Lap 010	0.361	0.226	0.516	**Lap 027**	0.497	-	0.412
Lap 011	0.160	0.335	0.329	**Lap 028**	0.338	-	0.276
Lap 012	-	0.195	0.474	**Lap 031**	0.253	-	0.313
Lap 014	0.349	0.116	0.315	**Lap 032**	0.128	-	0.074
Lap 015	0.289	-	-	**Lap 033**	0.042	-	-
Lap 017	0.222	-	0.525	**Lap 034**	0.343	-	0.212
Lap 018	0.224	-	0.555	**Lap 035**	-	-	-
Lap 019	0.227	-	0.120				

Overall peri-operative ACE transcription – mean of results from ACE 216 and ACE 2215 primer experiments, with all results normalised to GAPDH-3; **Sample ID** – sample identification number; **Pre-op** – Baseline pre-operative timepoint; **4 hours** – 4 hours post-operation; **24 hours** – 24 hours post-operation

10 week Post-operative ACE Transcription								
Sample ID	ACE 216			ACE 2215				Overall ACE IOD
	IOD (1)	IOD (2)	Mean IOD	IOD (1)	IOD (2)	IOD (3)	Mean IOD	
Lap 001	0.069	0.028	0.049	0.241	0.246	-	0.244	0.146
Lap 005	-	-	-	0.032	-	-	0.032	0.032
Lap 006	0.050	0.099	0.074	0.420	0.109	-	0.264	0.169
Lap 008	0.270	0.167	0.218	0.642	0.057	0.119	0.273	0.246
Lap 010	0.177	0.222	0.200	0.904	0.110	-	0.507	0.353
Lap 011	0.153	0.046	0.099	0.087	0.663	-	0.375	0.237
Lap 012	0.162	0.108	0.135	0.186	0.583	-	0.385	0.260
Lap 014	0.210	0.294	0.252	0.422	0.392	-	0.407	0.329
Lap 015	0.123	0.083	0.103	0.487	0.127	-	0.307	0.205
Lap 017	0.025	0.011	0.018	0.334	0.294	-	0.314	0.166
Lap 018	0.107	0.070	0.088	0.283	0.177	-	0.230	0.159
20 week Post-operative ACE Transcription								
Lap 001	0.448	0.148	0.298	0.192	0.270	0.277	0.246	0.272
Lap 005	0.341	0.701	0.521	0.039	-	-	0.039	0.280
Lap 006	0.056	0.093	0.074	0.445	0.160	-	0.303	0.188
Lap 008	0.363	0.428	0.396	0.615	0.324	0.587	0.509	0.452
Lap 011	0.365	0.117	0.241	0.307	0.629	-	0.468	0.355
Lap 012	0.340	0.146	0.243	0.530	0.254	-	0.392	0.317
Lap 015	0.410	0.635	0.522	0.906	0.285	-	0.595	0.559
Lap 017	0.063	0.028	0.046	0.672	0.458	-	0.565	0.305

Long term ACE transcription at 10 and 20 weeks following surgery – results from ACE 216 and ACE 2215 primer experiments, with all results normalised to GAPDH-3. Gaps due to poor RNA yield leading to lack of sample. **Sample ID** – sample identification number; **IOD (1-3)** – Integrated optical density, with experimental results from runs 1-3; **Mean IOD** – Mean integrated optical density from all runs; **Overall ACE IOD** – Overall mean integrated optical density from all runs and both primers

Appendix V

Protein expression

Peri-operative ACE Protein Concentration Results									
Sample ID	**Pre-operative**			**4 hours**			**24 hours**		
	Run 1 (ng/ml)	**Run 2 (ng/ml)**	**Mean (ng/ml)**	**Run 1 (ng/ml)**	**Run 2 (ng/ml)**	**Mean (ng/ml)**	**Run 1 (ng/ml)**	**Run 2 (ng/ml)**	**Mean (ng/ml)**
Lap 001	249	223	236	-	-	-	-	-	-
Lap 004	277	236	257	-	-	-	-	-	-
Lap 005	238	204	221	-	-	-	-	-	-
Lap 006	175	145	160	-	-	-	-	-	-
Lap 007	130	124	127	-	-	-	-	-	-
Lap 008	187	183	185	165	156	160	155	127	141
Lap 009	182	152	167	129	112	121	118	97	107
Lap 010	206	197	201	167	160	163	164	147	156
Lap 011	180	169	175	141	122	131	136	100	118
Lap 012	176	178	177	118	104	111	124	108	116
Lap 014	143	138	140	87	74	81	88	67	78
Lap 015	196	187	192	-	-	-	-	-	-
Lap 017	183	225	204	-	-	-	176	170	173
Lap 018	159	161	160	-	-	-	133	113	123
Lap 019	183	183	183	-	-	-	159	164	161
Lap 020	164	132	148	162	150	156	161	159	160
Lap 021	222	186	204	-	-	-	190	173	182
Lap 022	188	168	178	-	-	-	182	159	170
Lap 023	188	191	189	-	-	-	181	155	168
Lap 024	211	201	206	-	-	-	133	101	117
Lap 025	192	170	181	-	-	-	149	109	129
Lap 026	172	140	156	-	-	-	126	100	113
Lap 027	148	144	146	-	-	-	74	66	70
Lap 028	122	178	150	-	-	-	58	54	56
Lap 031	140	144	142	-	-	-	82	72	77
Lap 032	177	168	172	-	-	-	134	119	126
Lap 033	211	209	210	-	-	-	-	-	-
Lap 034	250	253	251	-	-	-	250	251	251
Lap 035	-	-	-	-	-	-	-	-	-

Peri-operative ACE protein concentration results from plasma – all results in ng/ml. Gaps due to patients refusing venesection leading to lack of sample. **Sample ID** – sample identification number; **Pre-op Runs (1-2)** – Baseline pre-operative timepoint experimental results from runs 1-2; **4 hours (1-2)** – 4 hours post-operation experimental results from runs 1-2; **24 hours (1-2)** – 24 hours post-operation experimental results from runs 1-2; **Mean** – Mean concentration from all runs at that timepoint

Peri-operative IL6 Protein Concentration Results									
Sample ID	**Pre-operative**			**4 hours**			**24 hours**		
	Run 1 (ng/ml)	**Run 2 (ng/ml)**	**Mean (ng/ml)**	**Run 1 (ng/ml)**	**Run 2 (ng/ml)**	**Mean (ng/ml)**	**Run 1 (ng/ml)**	**Run 2 (ng/ml)**	**Mean (ng/ml)**
Lap 001	0	0	0	-	-	-	-	-	-
Lap 004	0	0	0	-	-	-	-	-	-
Lap 005	0	0	0	-	-	-	-	-	-
Lap 006	0	4	2	-	-	-	-	-	-
Lap 007	20	43	32	-	-	-	-	-	-
Lap 008	0	0	0	108	106	107	34	33	34
Lap 009	4	0	2	491	469	480	168	136	152
Lap 010	0	0	0	307	354	330	14	14	14
Lap 011	0	0	0	141	140	141	5	6	6
Lap 012	0	0	0	163	150	157	34	35	34
Lap 014	0	0	0	195	197	196	92	88	90
Lap 015	0	0	0	-	-	-	-	-	-
Lap 017	0	0	0	-	-	-	3	6	5
Lap 018	0	0	0	-	-	-	119	128	123
Lap 019	0	0	0	-	-	-	110	113	112
Lap 020	30	26	28	30	26	28	16	15	15
Lap 021	0	0	0	-	-	-	99	97	98
Lap 022	21	24	22	-	-	-	42	43	42
Lap 023	0	0	0	-	-	-	129	111	120
Lap 024	0	0	0	-	-	-	131	122	126
Lap 025	7	26	17	-	-	-	120	120	120
Lap 026	0	0	0	-	-	-	326	304	315
Lap 027	0	0	0	-	-	-	285	276	281
Lap 028	0	0	0	-	-	-	131	103	117
Lap 031	0	0	0	-	-	-	341	301	321
Lap 032	0	0	0	-	-	-	64	41	53
Lap 033	0	0	0	-	-	-	-	-	-
Lap 034	0	0	0	-	-	-	134	112	123
Lap 035	-	-	-	-	-	-	-	-	-

Peri-operative IL6 protein concentration results from plasma – all results in ng/ml. **Sample ID** – sample identification number; **Pre-op Runs (1-2)** – Baseline pre-operative timepoint experimental results from runs 1-2; **4 hours (1-2)** – 4 hours post-operation experimental results from runs 1-2; **24 hours (1-2)** – 24 hours post-operation experimental results from runs 1-2; **Mean** – Mean concentration from all runs at that timepoint

Long Term ACE Protein Concentration

Sample ID	10 weeks			20 weeks		
	Run 1 (ng/ml)	Run 2 (ng/ml)	Mean (ng/ml)	Run 1 (ng/ml)	Run 2 (ng/ml)	Mean (ng/ml)
Lap 001	231	201	216	242	213	228
Lap 005	265	227	246	260	244	252
Lap 006	207	189	198	183	174	178
Lap 008	257	238	247	240	209	225
Lap 010	259	209	234	-	-	-
Lap 011	232	213	223	244	225	235
Lap 012	238	223	231	247	225	236
Lap 014	201	189	194	-	-	-
Lap 015	212	207	210	223	212	217
Lap 017	251	246	248	236	234	232
Lap 018	216	190	203	-	-	-

Long term ACE protein concentration results from plasma – all results in ng/ml. **Sample ID** – sample identification number; **10 weeks Runs (1-2)** – 10 weeks post-operation timepoint experimental results from runs 1-2; **20 weeks Runs (1-2)** – 20 weeks post-operation timepoint experimental results from runs 1-2; **Mean** – Mean concentration from all runs at that timepoint